

REGULATION OF CELL PROLIFERATION IN AUTOSOMAL DOMINANT  
POLYCYSTIC KIDNEY DISEASE

BY

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## Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is a life-threatening genetic disorder characterized by the presence of fluid-filled cysts primarily in the kidneys. Mutations in either the *PKD1* or *PKD2* genes are the underlying cause of ADPKD. It is a systemic disorder and some of the extra-renal manifestations include polycystic liver, cerebral aneurysms, cardiac valve abnormalities and hypertension. Progressive cyst formation and renal enlargement lead to renal insufficiency in these patients which need to be managed by life-long dialysis or renal transplantation. Epithelial cell proliferation and fluid secretion are two hallmark features of PKD.

Cux1 is a homeobox gene involved in cell cycle regulation during kidney development. In the developing mouse kidney, Cux1 is highly expressed in the nephrogenic zone where developing nephrons are present. Cux1 regulates the cell cycle by transcriptional repression of the cyclin dependent kinase inhibitors, p21 and p27, thereby increasing cell proliferation. As kidney development ceases, Cux1 is downregulated and adult kidneys show only low levels of Cux1. Cux1 is ectopically expressed in several mouse models of PKD, as well as, in ADPKD patients. Cux1 transgenic mice which overexpress Cux1 develops multiorgan hyperplasia including kidney hyperplasia, but they do not develop PKD. This suggests that the overexpression of Cux1 and the resultant increase in cell proliferation is not sufficient to cause PKD.

In this particular study, we addressed the hypothesis that Cux1 is required for cystogenesis and/or cyst progression in ADPKD. Results from our mouse model which carries a collecting duct specific deletion in the *Pkd1* gene (*Pkd1*<sup>CD</sup>) and a homozygous deletion in the *Cux1* gene shows that the complete loss of Cux1 results in slowing the cyst initiation process. Moreover, a reduction in the gene dosage of Cux1 in *Pkd1*<sup>CD</sup> mice led to a slow progression of

PKD resulting in the amelioration of the disease. The complete loss of the Cux1 gene and a reduction in its gene dosage mediated its effects by the de-repression of the p27 gene resulting in reduced cell proliferation. These studies point towards the importance of cell proliferation in the pathogenesis of PKD and show that Cux1 is required for cyst progression in ADPKD.



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## Dedication

She was my best friend, my confidante and much more than words can ever express. Losing her  
turned my world upside down.

*To my dearest mom who suffered an untimely death because of the complications of PKD.*

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## Chapter One: Introduction

### Polycystic Kidney Disease (PKD)

“Every one of us was once a cyst- a blastocyst”. Jared J. Grantham (1)

Polycystic kidney diseases are characterized by the presence of fluid-filled renal cysts and cysts in other epithelial organs (2). PKD patients show progressive cyst formation and massive renal enlargement that often leads to end-stage renal disease (ESRD) (3). Normal adult human kidneys make up about 0.5 per cent of a person's total body weight. A PKD patient who underwent bilateral nephrectomy after reaching ESRD had a total kidney weight of 22kg, which was around 21.6 per cent of his body weight (4). Normal renal tubules, which are approximately 40µm in diameter, enlarge to form cysts that measure centimeters in diameter (5). Cysts as large as 5cm were reported in PKD patients at their ESRD stage (6). PKD is inherited as an autosomal recessive (ARPKD) or an autosomal dominant (ADPKD) trait (2, 7).

#### ARPKD

ARPKD has an incidence rate of 1 in 20,000 live births (8, 9) and affects primarily neonates and children (2). ARPKD fetuses present with severe polycystic kidneys, biliary tract defects and oligohydramnios. Approximately half of the ARPKD neonates die shortly after birth due to respiratory insufficiency caused by pulmonary hypoplasia. In surviving patients, morbidity and mortality are mainly caused by systemic hypertension, renal dysfunction and portal hypertension that result from portal-tract hyperplasia and fibrosis (10). Cysts in these patients develop *in utero* and transient proximal tubular cysts are seen during early fetal development. However, the major manifestation is seen as fusiform dilatation of the collecting ducts (2). In ARPKD, cysts are attached and do not separate from the parental tubules (11).

Mutations in a single gene, *PKHD1*, which encodes a transmembrane protein known as fibrocystin/polyductin, causes ARPKD. Fibrocystin is present in cortical and medullary collecting ducts and biliary ducts. Subcellular localization of fibrocystin includes basolateral plasma membrane, primary cilia and the centrosome in renal epithelial cells (10, 11). Fibrocystin is thought to mediate terminal differentiation of the renal collecting ducts and intrahepatic biliary ducts (10).

## ADPKD

ADPKD is one of the most common hereditary disorders in humans. It has an incidence rate of 1 in 500 to 1 in 1000 individuals and occurs both in children as well as in adults (2). ADPKD occurs more frequently compared to other prominent genetic disorders. It is 10 times more common than sickle cell anemia, 15 times more common than cystic fibrosis, and 20 times more common than Huntington's disease. It is a systemic disorder which can have an adult as well as *in utero* onset (12). Extrarenal manifestations in ADPKD include cysts in other epithelial organs such as liver and pancreas. ADPKD patients also show connective tissue defects such as intracranial aneurysms, cardiac valve abnormalities, aortic dissection and abdominal wall hernias (13, 14). Hypertension is a very common symptom in ADPKD patients which often precedes the biochemical and clinical manifestations of the disease (15). Progressive loss of renal function is seen in ADPKD patients (7, 11). Renal enlargement in these patients leads to the displacement of other organs and with considerable renal enlargement, ADPKD patients look chronically pregnant. These patients account for approximately 10% of ESRD cases (7, 11). ADPKD is also the third leading cause of ESRD requiring dialysis and transplantation in the United States. Medicare costs for treating ESRD in ADPKD patients were found to exceed \$200 million per year according to a report in 1993 (12).

## ADPKD genes

ADPKD is genetically heterozygous. Mutations in the *PKD1* gene located on chromosome 16p13.3, causes ADPKD in 85% patients. The remaining 15% patients with ADPKD have mutations in the *PKD2* gene which is located on chromosome 4q21-23 (2, 16). It is uncertain if there is a third gene associated with ADPKD as some patients do not show linkage to either the *PKD1* or *PKD2* genes (16, 17). Identical renal and extrarenal manifestations are seen in patients with *PKD1* or *PKD2* mutations. Nevertheless, *PKD2* patients show a later onset, have longer renal survival and present fewer complications compared to *PKD1* patients (2).

The *PKD1* gene is a very large gene and has 46 exons within the 52 kb genomic DNA. A region in the *PKD1* gene which spans from exon 1 to 33 is duplicated at six other sites on the same chromosome. Mutation analysis of the *PKD1* gene has been difficult owing to these duplicated regions (2). The *PKD1* gene has some unusual structural features such as a high GC content and multiple simple repeats. Of interest, a 2.5kb polypyrimidine tract is seen in intron 21, which may interfere with its replication, transcription and RNA processing (11). The *PKD2* gene is comparatively smaller than the *PKD1* gene and is 25% homologous to a region of the *PKD1* gene (2). Screening of the ADPKD population has discovered 864 *PKD1* and 139 *PKD2* germ-line mutations to date (pkdb.mayo.edu). Most of these mutations produce truncated protein products due to nonsense changes, splicing defects, frame-shift deletion or frame-shift insertions. However, some missense and in-frame mutations have also been described (17). Protein products of the *PKD1* and *PKD2* genes are collectively called polycystins. A detailed account of polycystins is given in a later section.

ADPKD- a developmental disorder

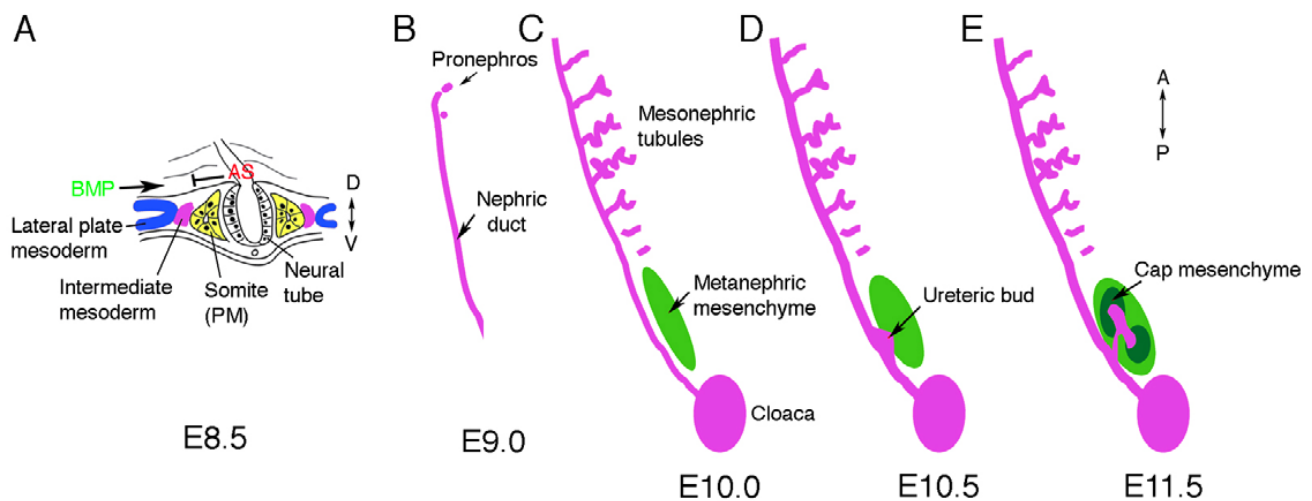
The onset of symptoms in ADPKD does not become apparent until the fourth or fifth decade of life in an ADPKD patient. However, numerous evidence suggests that cystogenesis occurs as early as *in utero* in these patients. Even with an ultrasound detection threshold of >7.0mm, cysts have been detected at birth in ADPKD patients (18). Cysts have also been detected in stillborn fetuses, live born babies immediately after birth and in infants (19). A study measuring the growth rates of individual cysts in adult ADPKD patients over a period of 3 years suggests that cysts that are detected in newborn patients must have grown at exuberant rates *in utero* and at slower rates thereafter (18).

## Mammalian kidney development

The nephron, which is the functional unit of the excretory system in vertebrates, has similar characteristic features in lower vertebrates and also in higher vertebrates such as mammals (20). As it develops, the mammalian kidney goes through three spatially and temporally different stages known as the pronephros, the mesonephros and the metanephros (20-23). The mammalian kidney originates from the intermediate mesoderm (IM) which lies in between the axial and the lateral plate mesoderm (24, 25). The different stages of mammalian kidney development in a mouse embryo are shown in figure 1-1.

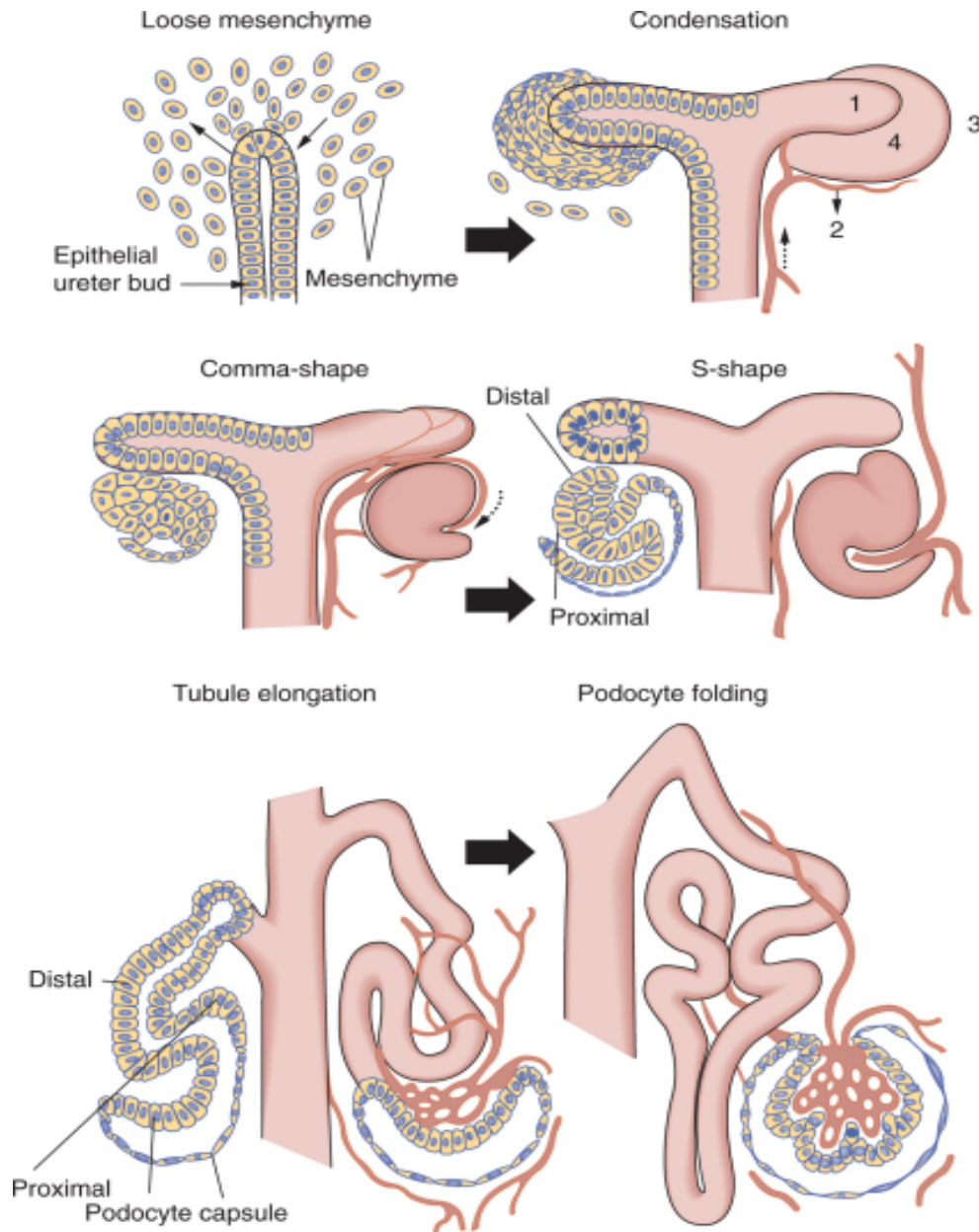
As the first step in mammalian kidney development, the primary nephric duct, also known as the Wolffian duct or the pronephric duct, arises from the IM and grows in the rostro-caudal direction (24, 26). The elongating pronephric duct induces the formation of two sets of tubules in the adjacent undifferentiated mesoderm. In the mouse embryo, these inductive events take place at embryonic day 8 (E8) and E10 to produce the pronephros and the mesonephros,

respectively. The pronephric tubules are rudimentary structures and do not have any functional role in the mouse embryo. However, the caudal mesonephric tubules are well developed with glomeruli and convoluted proximal tubule-like structures and serve as transient filtration units which empty into the nephric duct (24-26). Mesonephros also gives rise to the ductal system of male reproductive system in mammals. In humans, the pronephros can be seen by E22 and the mesonephros by E24 (22). The mesonephros degenerates as the definitive metanephric kidneys form in mammals (24).



**Figure 1-1: Stages of kidney development in the mouse embryo.** A: Schematic cross section of a mouse embryo at E8.5. Kidneys arise from the intermediate mesoderm (IM, pink) which lie between the lateral plate (blue) and the paraxial mesoderm (PM, yellow). B-E: Schematic diagrams of different developing kidney structures in the anteroposterior (AP) axis in a mouse embryo. B: The pronephric duct arises from the intermediate mesoderm in the mouse embryo at E9.0. C: The nephric duct grows caudally until it reaches the cloaca. Mesonephric tubules can be seen at E10.0. The mesonephric tubules which are found more caudally, are more developed compared to the proximal ones. Posterior cells of the intermediate mesoderm specialize to form an aggregate called metanephric mesenchyme (MM, green). MM gives rise to all the segments of the nephron. D: Ureteric bud (UB), an outgrowth from the nephric duct invades the MM by E10.5. UB gives rise to the collecting system of the kidneys. E: The UB bifurcates and the induced mesenchyme, known as the cap mesenchyme surrounds the tips of the UB. Reproduced with permission from Dressler, 2009 (25).

The functional kidney in adult mammals- the metanephros, forms at the posterior end of the IM (25). An outgrowth of the primary nephric duct called ureteric bud (UB) extends into the surrounding specialized region of the IM known as the metanephric mesenchyme (MM). The MM contains the progenitor cells of nephrons, the functional units of the adult kidney. The MM also provides inductive signals for the primary nephric duct to invade and form the UB at its caudal end. Thus, the epithelial cells of the UB invade the MM and undergo branching morphogenesis to generate the collecting system of the kidneys. At the same time, mesenchymal cells from the MM aggregate around the tips of the newly formed UB branches and begin a mesenchyme-to-epithelial conversion (MET). This process is called nephrogenesis as it generates the nephron epithelia (24, 27). During nephrogenesis, the mesenchymal aggregates that form around the UB go through three different stages known as the polarized renal vesicle, the comma-shaped body and the S-shaped body. One end of the renal vesicle remains in contact with the UB epithelium and two clefts are formed in the renal vesicle, one after the other, to form the comma and the S-shaped bodies, respectively. The distal end of the S-shaped body that remained in contact with the UB fuses with it to form a single, continuous epithelial tubule. Subsequently, endothelial cells invade the more proximal cleft in the S-shaped body and form the glomerular tuft. The ureteric bud branches continuously, inducing new nephrons along the radial axis of the kidney. By virtue of this process, the oldest nephrons are found close to the medullary region whereas the youngest nephrons are located more peripherally in the nephrogenic zone in a developing kidney (24). In the mouse embryo, metanephric induction starts at E10.5 and nephrogenesis continues until a week after birth (25, 28). In humans, the metanephros begins development at around E35 to E37 and nephrogenesis ceases at approximately 34 weeks of gestation (22, 18). The different stages in nephrogenesis are shown in figure 1-2.



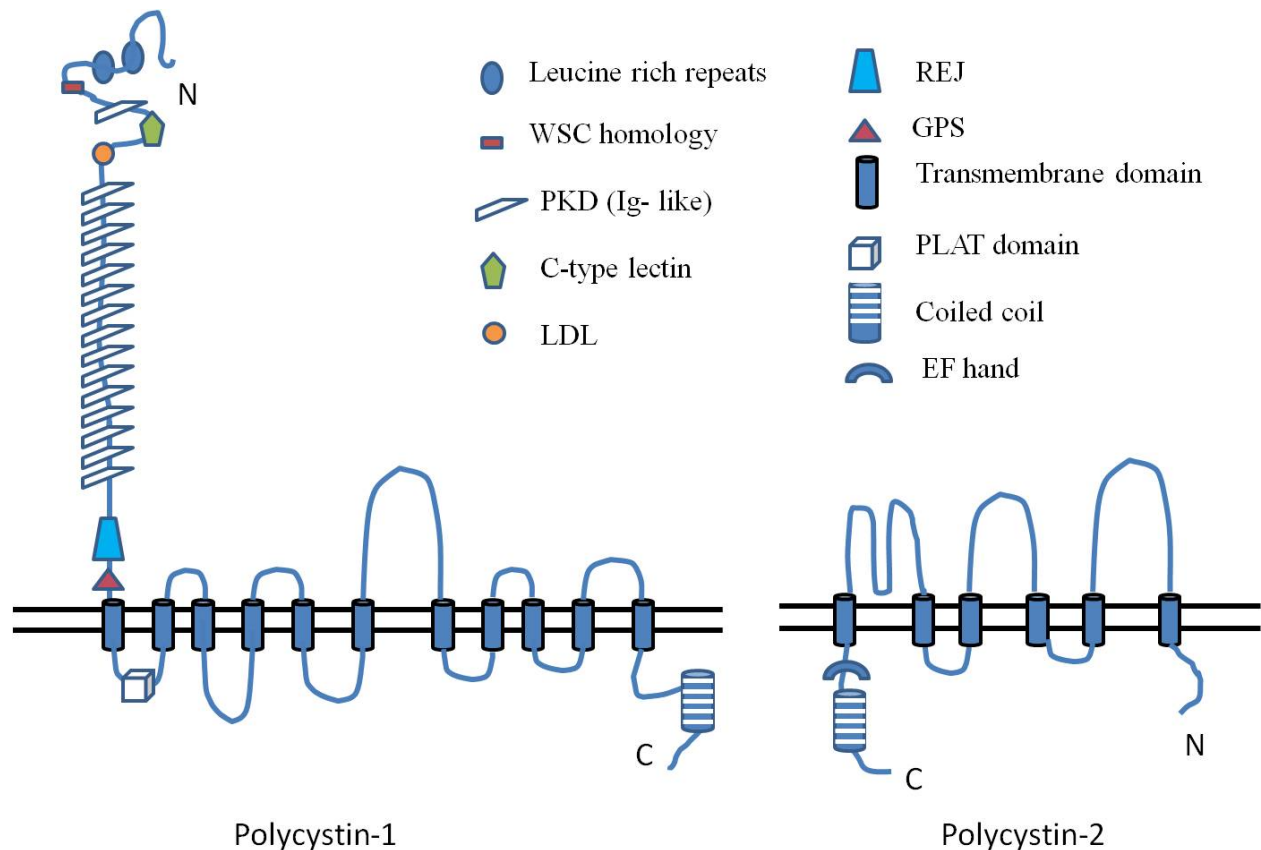
**Figure 1-2: Stages of nephrogenesis** The adult metanephric kidney in mammals develops by a reciprocal interaction between the epithelial UB and the MM. During the condensation stage, the loose mesenchymal cells of the MM condense around the tips of the epithelial UB. The mesenchymal cells undergo mesenchyme-to-epithelial conversion (MET). After the condensation stage, the induced mesenchyme forms the comma and S-shaped structures. Later, the tubules elongate and the podocytes in the glomeruli fold to give rise to a mature nephron. Reproduced with permission from Brenner and Rector's, *The Kidney* (130). This figure was originally published in reference 132.

## Polycystins, their expression pattern and functions

Polycystin-1 (PC1), protein product of the *PKD1* gene, is an integral membrane protein with a large extracellular N-terminal domain (~3000 amino acids), eleven transmembrane domains (~1000 amino acids) and a small C-terminal cytosolic domain of about 200-225 amino acids (2, 29). The extracellular domain of PC1 contains a region which is homologous to the sea urchin protein, receptor for egg jelly or REJ. Regulation of ion transport in the sea urchin sperm involves the REJ module of the REJ protein. Likewise, the REJ domain in PC1 is also thought to be involved in ion transport (30). The PC1 extracellular domain also contains several other domains such as leucine-rich repeats (LRRs), a C-type lectin domain, LDL-A region and multiple Ig-like domains or PKD domains, all of which are implicated in cell-cell or cell-matrix interactions (2, 11, 14, 31). The first cytoplasmic loop of PC1 contains a lipoxxygenase domain (PLAT domain named after Polycystin-1, lipoxxygenase and alpha toxin) which is essentially a  $\beta$ -sandwich domain. PLAT domains are usually involved in protein-protein or protein-lipid interactions, and in PC1, the presence of PLAT domain may indicate its interaction with other proteins (32).

The cytoplasmic tail of PC1 is found to bind heterotrimeric G proteins *in vitro*, activate the AP-1 transcription factor and take part in the regulation of Wnt signaling (33). The cytoplasmic tail contains a coiled-coil domain with which it interacts with polycystin-2 (PC2), the protein product of the *PKD2* gene (11). A sequence motif that is rich in proline, glutamic acid, serine and threonine (PEST), which facilitates ubiquitin-mediated degradation, is also seen in the C-tail of PC-1 (34). The PC1 C-tail also contains a putative nuclear localization sequence (NLS) that consists of two stretches of basic amino acid residues (35).





**Figure 1-3: Structure of polycystins.** Polycystin-1 has a large extracellular domain, 11 transmembrane domains and a short cytoplasmic C-terminal tail. The coiled coil domain in the C-terminal end of PC1 interacts with the C-terminal tail of Polycystin-2. Polycystin-2 has cytoplasmic N and C-terminus. Together, PC1 and PC2 mediate calcium entry into cells. Redrawn from references 2 and 11.

PC1 is widely expressed in organs such as kidney, liver, brain, pancreas, small intestine, lungs and heart (40). PC1 is present on the plasma membrane at focal adhesions, desmosomes, tight junctions and adherens junctions and also in the shaft and basal body of primary cilia (2, 16, 36). PC1 is also expressed in urinary exosomes, which are small vesicles (50-100nm) secreted by the renal epithelial cells into the urine. Normal urine shows the presence of thousands of proteins

and most of these proteins are packaged into exosomes and shed into the urine. Exosomes are products of the multivesicular body sorting pathway (MVB). The MVB-sorting pathway consists of the endocytosis of the integral membrane proteins to form endosomes, fusion of these endosomes with the MVB and finally fusion of the MVB with the apical plasma membrane to release exosomes (37, 38).

PC2, the protein encoded by the *PKD2* gene, is also a membrane associated protein with six transmembrane domains and cytoplasmic N- and C- terminal domains. PC2, also known as TRPP2, is a member of the family of transient receptor potential (TRP) ion channels. It is a non-selective cation channel permeable to  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  ions. The channel activity of PC2 is modulated by a rise in the intracellular calcium levels. PC2 is insensitive to two other ligand-gated calcium channels, IP3 receptors and ryanodine receptors and hence is proposed to be a third class of calcium release channels in addition to the other two. PC2 is shown to form heteromeric channels with two other TRP family members, TRPC1 and TRPV4 (36).

PC2 is found on the apical and basolateral plasma membrane, endoplasmic reticulum, Golgi, shaft and basal bodies of the primary cilia, and also in urinary exosomes (11, 16, 36). The trafficking of PC2 among the ER, Golgi and plasma membrane is modulated by the phosphorylation of an acidic cluster present on its C-terminal tail by casein kinase II (36). Most of the cellular pool of PC2 is found in the intracellular compartments where it modulates the release of calcium from intracellular stores. There are at least two cytoplasmic domains in PC2, one in the N-terminus and the other in the C-terminus that contribute to the oligomerization of PC2. The C-terminus of PC2 contains a coiled-coil domain with which it interacts with PC1, an EF hand characteristic of calcium binding proteins and an ER retention signal that localizes PC2 to the ER and Golgi compartments (34).

## Expression of polycystins during kidney development and in ADPKD

The expression pattern of polycystins in the developing human fetal kidneys between the gestational ages of 13- 40 weeks has been described (31, 39). PC1 and PC2 are weakly expressed in early nephrogenic precursors such as comma and S-shaped bodies. Weak but detectable expression of the polycystins was also seen in the proximal and distal branches of the ureteric bud. Maturing proximal and distal tubules and collecting ducts showed a marked level of expression of the polycystins. While proximal tubules sustained polycystin expression only until 28 weeks of gestation, both cortical and medullary collecting ducts maintained the expression pattern until 40 weeks of gestation. Analysis of adult human kidneys also showed a weak expression pattern of polycystins in the proximal tubules. Continued expression and colocalization of PC1 and PC2 were seen in the medullary collecting ducts (39).

The expression pattern of murine PC1 was analyzed by Geng *et al.* Similar to the expression profile of PC1 in humans, murine PC1 was widely expressed in the kidneys. It was also expressed in other organs such as brain, liver, pancreas, small intestine, lungs and heart. Developing kidneys showed the weakest expression of PC1 in the ureteric bud tips which suggest that PC1 may not be required for nephrogenic induction. Ureteric bud derivatives such as collecting ducts, papillary ducts and renal pelvis show a strong expression of PC1 during later developmental stages. This tubular expression of PC1 continued after birth and was maintained until the third week postnatally after which PC1 expression becomes undetectable (40).

Murine embryonic ectoderm and endoderm shows the presence of PC2 by E6.0. PC2 is widely expressed in the embryonic stages, while its expression becomes more restricted after birth. By E12.5, metanephric ureteric bud showed low-intensity staining for PC2. Proximal

tubules showed a weaker staining, while distal tubules strongly express PC2 by E15.5. Medullary collecting ducts showed the expression of PC2 by postnatal day 14 (41).

Ward *et al* described the expression pattern of PC1 in adult human tissues, in ADPKD kidneys and also in polycystic liver (31). Quantification of PC1 expression in adult human tissues showed highest expression in brain, lowest in thymus and an intermediate level of expression in the kidneys. This widespread expression of PC1 in a variety of tissues may explain the systemic nature of ADPKD. End-stage ADPKD kidneys and an ADPKD polycystic liver analyzed, showed an approximately 2-fold increase in the Polycystin-1 mRNA expression level compared to the normal kidneys and livers respectively (31).

#### Polycystins as mechanosensors

ADPKD, together with many other diseases that present with renal cysts, was inducted into a family of diseases known as ciliopathies. This came after the discovery that many cystoproteins that cause renal cysts localize to the once disregarded organelle, the non-motile primary cilia. Primary cilia are sensory organelles which protrude from the center of epithelial cells. They grow out from the basal bodies or centrosomes and are microtubule-based structures. Primary cilia are highly conserved in evolution and are used to detect a wide variety of physical and chemical stimuli that are of mechanical, photonic, olfactory or hormonal in nature (42).

The association between cilia and PKD was first discovered when mutations in *Tg737*, responsible for renal cystic disease in the *orpk* mouse model of PKD, showed stunted primary cilia in their renal epithelial cells. The orthologue of *Tg737* in *Chlamydomonas*, *Ift88*, is required for intraflagellar transport and assembly of motile cilia. Subsequently it was shown that polycystins and fibrocystins also localize to the primary cilia (42).

Renal tubular epithelial cells, including all the segments of the nephron and the collecting ducts, show the presence of a single primary apical cilium (13). Fluid flow in renal tubular epithelial cells bends the primary cilium on their surface. PC1 present on the cilium is thought to sense the fluid flow with its large extracellular domains, in turn activating the associated PC2 calcium channels. This allows calcium influx which leads to a calcium induced calcium release from other intracellular  $\text{Ca}^{2+}$  stores leading to various genetic changes in cells (16, 43). A model for polycystins signaling through primary cilia is shown in figure 1-4 B.

A flow- $\text{Ca}^{2+}$  imaging system was used to test the functionality of PC1 on primary cilia. Mouse embryonic kidney cells responded to fluid flow at a rate comparable to the physiological urine flow rate. However, Pkd1 null cells failed to respond to fluid flow rates higher or lower than those detected by wild type cells confirming that PC1 is required to detect fluid flow. Atomic force microscopy studies revealed that the PKD domains on PC1 exhibit remarkable mechanical strength, further supporting the mechanosensory function of PC1 (36).

Other diseases such as nephronophthisis (NPHP), Bardet-Biedl syndrome (BBS) and Orofaciodigital syndrome also present with renal cysts, and the protein products of the genes mutated in these diseases all localize to the cilia, basal bodies or centrosomes. In fact, protein products of all the genes that cause renal cystic diseases in humans, mice and zebrafish localize to the primary cilia, basal bodies or centrosomes placing primary cilia at the center of cystic kidney diseases. However, since the cystoproteins also localize to many other sub-cellular locations, a ciliary defect alone cannot be attributed as a primary defect in these diseases. Nevertheless, a systemic nature of different types of PKDs, BBS and NPHP favors the ciliary hypothesis because of the presence of cilia in many of the organs affected in these diseases (42).

## Channel activity of polycystins

The last six transmembrane domains of PC1 share significant sequence homology with the domains of Na<sup>+</sup> and Ca<sup>2+</sup> channels and transient receptor potential (TRP) channels. However, by itself PC1 cannot form ion channels since PC1 overexpression alone does not yield any measurable channel activity (36).

In response to local increases in intracellular calcium, PC2 releases calcium from intracellular stores. The loop between the fifth and the sixth transmembrane domains is involved in the calcium conducting activity of PC2. The third transmembrane domain is also involved partially in this process. In addition to its direct involvement in calcium induced calcium release, PC2 also indirectly regulates calcium levels in the cell by interacting with two intracellular calcium channels, ryanodine receptors and IP3 (inositol 1,4, 5-triphosphate receptor) receptors. PC2 inhibits ryanodine receptor mediated calcium release by binding to the channel in its open state thereby decreasing its conductance. Direct binding between the C terminus of PC2 and IP3R modifies IP-3 induced calcium flux (34).

An interaction between PC1 and PC2 generates a functional ion channel. It is not certain whether this is through the activation of PC2's intrinsic channel properties, or due to the formation of a functional complex between these two proteins (34). Owing to the calcium channel activity of polycystins, genetic mutations in any of the polycystins disrupt intracellular calcium regulation leading to abnormal cell proliferation. A study measuring the steady-state intracellular calcium levels in normal and ADPKD renal cell cultures found that calcium levels in ADPKD cells are 20nM lower than in normal renal cells (44).

## Other functions of polycystins

PC1 found on the plasma membrane may interact with PC2 present on the adjacent endoplasmic reticulum (16). PC1 may also act as a receptor for a ligand which is yet to be identified. Upon receiving a stimulus, PC1 may signal to the interior of the cell by its interaction with PC2 on the plasma membrane. This signaling event can result in the activation of calcium channels, an increase in cytoplasmic calcium which leads to exocytosis and changes in gene expression (Figure 1-4 A) (2).

Cellular signaling pathways modulated by the polycystins can be broadly classified into three categories: G- protein activation, growth regulation and Wnt pathway modulation (34).

### G-protein activation

PC1 functions as a G-protein coupled receptor (GPCR) which can regulate cell proliferation, fluid secretion, cell polarity and differentiation (Figure 1-3 C and D) (2). PC1 contains sequences that are found in GPCRs. These include a proteolytic site domain and a polybasic domain found in its C-terminal tail, which has been shown to activate Gi/G0 proteins *in vitro*. However, PC1 is an atypical GPCR since it has 11 transmembrane domains in contrast to 7 transmembrane domains found in typical GPCRs. PC1 has been shown to bind and stabilize the regulator of G-protein signaling 7 (RGS7), member of a family of proteins which are capable of accelerating the hydrolysis of GTP bound to the G $\alpha$  subunit of G-proteins. Moreover, RGS7 was shown as a candidate modifier gene in a mouse model of PKD (29).

Downstream signaling through GPCRs can lead to the activation of c-Jun N-terminal kinase (JNK) and activator protein-1 (AP-1) pathways. The JNK/AP-1 pathway controls various cellular processes such as cell cycle regulation, cell growth, differentiation, apoptosis and

inflammation. In *Drosophila*, JNK signaling is involved in the developmental regulation of planar polarity, epidermal adhesion and integrity (29). Many of these pathways controlled by JNK/AP-1 are also involved in the pathogenesis of PKD.

Arnould *et al* demonstrated the activation of the JNK/AP-1 pathway via the PC1 C-terminal tail. Several components of this pathway such as protein kinase C  $\alpha$  (PKC  $\alpha$ ) and small G proteins such as Cdc42 and Rac1 were involved in mediating this signal (45). Parnell *et al* showed that the activation of JNK/AP-1 pathway is mediated by signaling through G $\alpha$  and G $\beta\gamma$  subunits (29). Moreover, activity of the AP-1 components such as ATF-2, c-jun and c-fos were increased in ADPKD patients and in a hypomorphic *Pkd1* mouse model further suggesting the involvement of JNK/AP-1 pathway in PKD pathogenesis (46).

Additional evidence for PC1 signaling through G-proteins comes from the observation that PC1 activates phospholipase C (PLC), an event mediated by the G $\alpha_q$ . This leads to the subsequent activation of the calcineurin/ NFAT (nuclear factor of activated T-cells) pathway. Signaling through this pathway also relates to the function of polycystins as regulators of intracellular calcium levels. Exogenous expression of the PC1 C-tail results in an increase in calcium levels in a reaction requiring PLC  $\beta$ . This intracellular increase in calcium leads to the activation of calcineurin, a serine-threonine phosphatase which dephosphorylates NFAT. Activated NFAT translocates to the nucleus and regulates target genes at composite NFAT/AP-1 elements. In addition to the evidence for PC1 mediating NFAT activation, NFAT was also shown to be expressed in renal tubular epithelial cells of developing and adult mice which also correlated with the temporal expression of PC1 in these cells. This suggests that NFAT and PC1 may work together in a pathway (47).



## Growth regulation

In accordance with the increased cell proliferation defects seen in PKD, PC1 and PC2 are directly involved in regulating the cell cycle. One of these mechanisms includes signaling through the JAK-STAT pathway (Figure 1-4 E-F). Signaling through this pathway leads to the activation of STAT1 and STAT3, subsequent upregulation of a cyclin dependent kinase inhibitor (CKI) p21 and the inhibition of cyclin dependent kinase 2 (cdk2) which ultimately leads to cell cycle arrest at the G0/G1 transition (Figure 1-4 G) (2, 48).

Another evidence for a direct role of polycystins in the cell cycle was shown by the direct interaction of PC2 with a helix-loop-helix (HLH) protein Id2, which regulates cell proliferation and differentiation. Phosphorylation of PC2 by PC1 leads to its interaction with Id2. Id2 is found in a complex with another HLH protein, E47, which transcriptionally activates p21. Id2 lacks a nuclear localization signal and can be transported to the nucleus only through its interaction with E47 or some other proteins. Interaction of the Id2-E47 complex with PC2 sequesters this complex outside the nucleus. When PC2 is unable to bind to this complex, Id2 is translocated to the nucleus and it exerts its dominant negative effects on E47 and other HLH proteins. In this manner, normal expression of PC1 and PC2 leads to cell cycle arrest by an increase in p21, whereas a mutation in one of the polycystins leads to the dysregulation of this pathway resulting in increased cell proliferation (49).

There are three proteolytic cleavage sites present on PC1. PC1 undergoes partial cleavage at the extracellular GPS domain, however, the N- and C-terminus of the protein remains non-covalently linked. A missense mutation at the GPS domain, which makes PC1 non-cleavable has been reported in ADPKD in patients. Moreover, a mutant PC1 that cannot undergo GPS cleavage

was unable to rescue PC1-null cultured cells. This evidence suggests the requirement of PC1 to be cleaved at the N-terminal GPS domain to be fully functional. However, not all the PC1 molecules in a cell are cleaved; they exist in a heterogeneous population of full length and GPS cleaved isoforms (11, 34).

Cleavage of PC1 in the C-terminal tail (CTT) which releases a soluble ~ 35kD fragment was described by Chauvet *et al.* This C-terminal cleavage suggested that PC1 may be involved in successive cleavage events similar to that of the regulated intramembrane proteolysis (RIP) pathway. In the RIP model described for various cell surface receptors such as Notch, APP, E-cadherin, ErbB4 and CD44, the cytoplasmic segment of a transmembrane protein enters the nucleus after its activation and modulates gene expression, bypassing adaptor proteins and kinase/phosphatase cascades (35). Chauvet *et al* also showed that this nuclear translocation of the PC1 CTT leads to the activation of the AP-1 pathway. PC2 seems to keep PC1 CTT from not entering the nucleus since co-transfection experiments using full length PC2 and PC1 CTT led to the retention of PC1 CTT outside the nucleus and a reduction in AP-1 activity. PC2 is also required to interact with PC1 CTT to exert its inhibitory effect on PC1, since co-transfection experiments with a mutant PC2 which could not interact with PC1 CTT led to a reduction in AP-1 activity. Unilateral ureteral ligation (leading to a reduction in fluid flow) and inactivation of the *Kif3a* gene (resulting in the loss of the cilia where polycystins are localized) in mice led to an increase in the nuclear translocation of PC1 CTT. These results showed that PC1 CTT cleavage and nuclear translocation are associated with the mechanosensory effects of cilia. A reduction in fluid flow or loss of fluid flow sensitivity in renal epithelial cells can lead to an accumulation of PC1 CTT in the nucleus and transcription of its target genes (35). PC1 with its long extracellular domain is assumed to detect fluid flow in renal epithelial cells and PC2 has an inhibitory effect

on the translocation of PC1 CTT. Hence, mutations in any of the polycystins can render renal epithelial cells insensitive to fluid flow and lead to the increased transcription of its target genes.

Low *et al* described a more distal cleavage in the C-terminal domain of PC1 which generates a ~15-17kDa CTT fragment. The PC1 CTT fragment interacts with P100, a coactivator protein. The P100 protein has also been localized to the shaft and the basal bodies of renal cilia where PC1 is also present. P100 is overexpressed in the cyst-lining cells of ADPKD patients. The PC1 CTT fragment, together with P100, binds to the transcription factor STAT6 and leads to an enhancement of STAT6 dependent transcription. STAT6 is localized to the nucleus in MDCK and renal epithelial cells, however, subjecting cells to a constant flow of fluid (similar to the fluid flow in renal tubules) drastically changes the translocation of STAT6 from the nucleus to the primary cilia. Moreover, high levels of nuclear STAT6 were seen in the cyst-lining cells of ADPKD patients indicating that STAT6 dependent gene expression is upregulated in ADPKD. Under normal fluid flow conditions, PC1 keeps STAT6 sequestered to the cilia together with P100 preventing the transcription of P100/STAT6 genes. “No flow” conditions lead to the cleavage of the PC1 tail which translocates to the nucleus with P100 and STAT6 to mediate gene transcription. No flow conditions have been observed in renal injury as well as in the cysts of PKD. Dilated tubules in PKD also show decrease of fluid flow (50).

A recent observation by Talbot *et al* shows the mechanism by which PC1 regulates STAT activity. Membrane anchored PC1 CTT activates STAT3, however, activation of STAT1 and 6 requires soluble PC1 tail as well as a previous activation of these STATs by cytokine signaling (coactivation) (Figure 1-4 E-F). They found that STAT3, in addition to being activated by membrane-anchored PC1 CTT, can also be coactivated by the soluble PC1 CTT. The coactivation process can lead to an exaggerated cytokine response. STAT3 activity is highly

upregulated in ADPKD and PKD mouse models, while it is downregulated in normal renal epithelial cells after differentiation (51).

The mTOR (mammalian target of rapamycin) pathway which regulates protein translation, cell proliferation and cell growth has been shown to be a target for PC1. Shillingford *et al* showed that PC1 CTT interacts with tuberin, the protein product of the TSC2 gene. Mutations in TSC2 lead to tuberous sclerosis which is characterized by the presence of hamartomas (benign tumor-like malformations) in multiple organs and renal cysts. Tuberin regulates the kinase activity of mTOR through a small GTPase, Rheb. Activated mTOR phosphorylates and activates its downstream effectors- S6K1, S6K2 (ribosomal kinases) and 4E-BP1 and 4E-BP2 (eukaryotic initiation factor 4E-binding proteins) that leads to the stimulation of protein synthesis and proliferation. Shillingford *et al* also provided evidence for the increased activity of the mTOR pathway in the cyst-lining epithelial cells of ADPKD patients by showing an increase in activated phosphorylated mTOR and S6 kinase. Moreover, rapamycin, an immunosuppressant drug which is also a specific inhibitor of the mTOR pathway, was able to alleviate the cystic phenotype in two different PKD mouse models. These mouse models and a rat PKD model in which mTOR inhibition slowed the progression of PKD did not have a primary mutation in polycystin genes. This suggests that the activation of the mTOR pathway may be common to all renal cystic diseases despite their primary germ-line mutation (52, 53).

In a recent report, Dere *et al* showed that PC1 regulates mTOR activity by altering the subcellular localization of tuberin. Suppression of mTOR by tuberin takes place only when tuberin is tethered to the membrane. Phosphorylation of tuberin by PI3K/AKT leads to its binding by 14-3-3 adaptor protein. This leads to the partitioning of tuberin to the cytosol thereby making it unavailable to bind with Rheb and its activating partner, TSC1. In the presence of a

functional membrane bound PC1 CTT, phosphorylation of tuberlin by PI3K/AKT is inhibited. This leads to the tethering of TSC2 protein to the membrane and repression of mTOR signaling. In contrast, mTOR pathway is activated in the absence of a functional PC1 (52, 53).

#### Wnt pathway modulation

The Wnt signaling pathway, which is involved in embryonic induction, generation of cell polarity, cell proliferation and the specification of cell fate, is regulated by PC1. Wnts are secreted glycoproteins and canonical Wnt signaling regulates the cellular levels of a multifunctional polypeptide,  $\beta$ -catenin. The presence of Wnt ligands leads to the stabilization, accumulation and nuclear translocation of soluble  $\beta$ -catenin. Secreted Wnts bind to frizzled receptors on the cell surface, activating disheveled proteins and inhibiting glycogen synthase kinase (GSK-3 $\beta$ ). Once inhibited, GSK-3 can no longer direct  $\beta$ -catenin for proteasomal degradation which leads to the accumulation and nuclear translocation of  $\beta$ -catenin. Once inside the nucleus,  $\beta$ -catenin interacts with the TCF/LEF family of transcription factors to regulate gene expression (34, 54, 55).

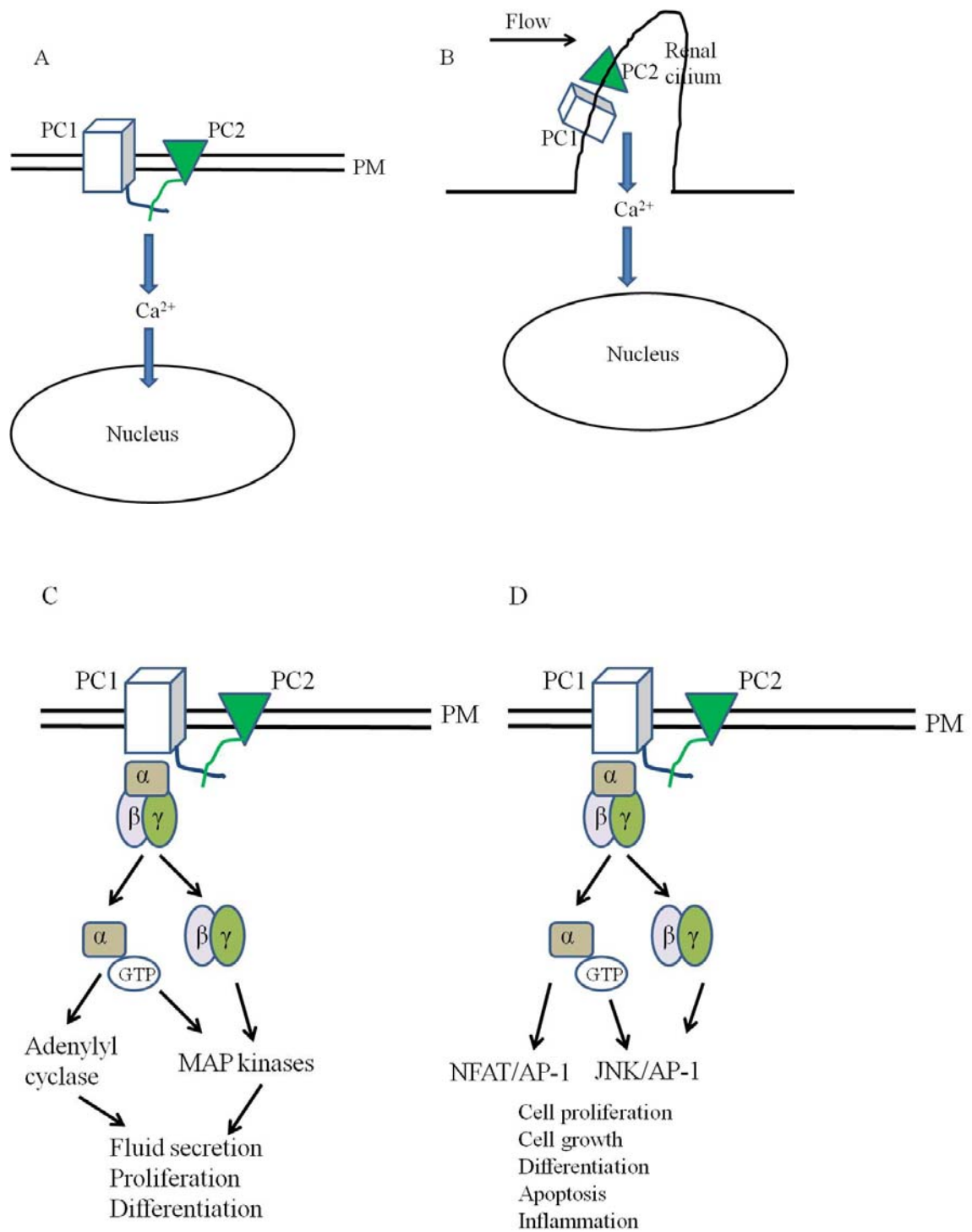
Expression of stabilized  $\beta$ -catenin in renal tubular cells leads to the development of PKD in animals (54). Microarray analysis shows evidence for the activation of Wnt signaling in the cyst-lining cells of ADPKD patients (55). Experiments performed to demonstrate Wnt modulation by the PC1 C-tail has generated contradictory results. In contrast to previous experimental results which showed that the PC1 C-tail activates canonical Wnt signaling (54), a recent report suggest that PC1 C tail physically associates with  $\beta$ -catenin and acts as an inhibitor of intracellular Wnt signaling (55). Disparity between these results has been attributed to the use of membrane anchored PC1 CTT in the previous report versus the use of a soluble PC1 CTT in

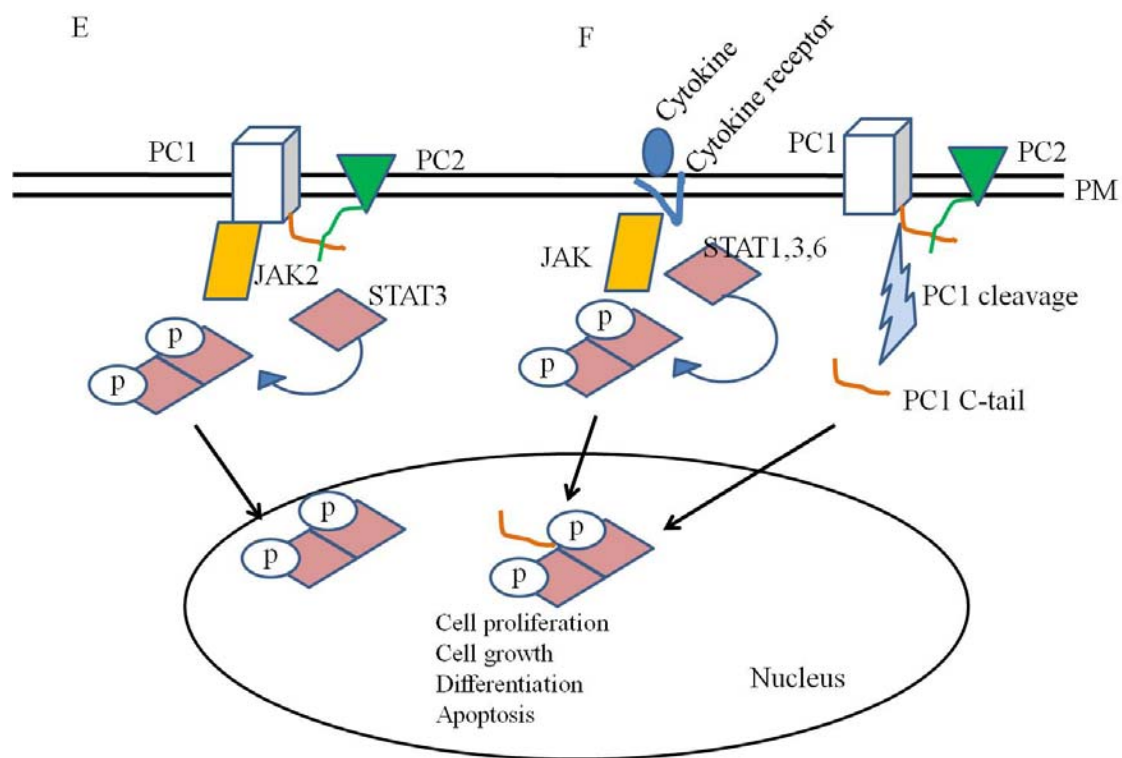
the recent report. PC1 exerts this inhibitory effect on Wnt signaling pathway by reducing the apparent affinity of the interaction between  $\beta$ -catenin and TCF. The inhibitory effect of the PC1 CTT on canonical Wnt signaling also adds up to the observation that the overexpression of c-myc, a  $\beta$ -catenin/ TCF regulated oncogene, induces a renal cystic phenotype in transgenic mice. c-myc is also overexpressed in ADPKD and several other renal cystic diseases (55).

Expression of a mutant PC2 in cell culture resulted in an increase in  $\beta$ -catenin protein levels. However, it is not certain whether this effect is mediated directly by the inactivation of PC2 or through an indirect effect that PC2 might have on PC1 (34).

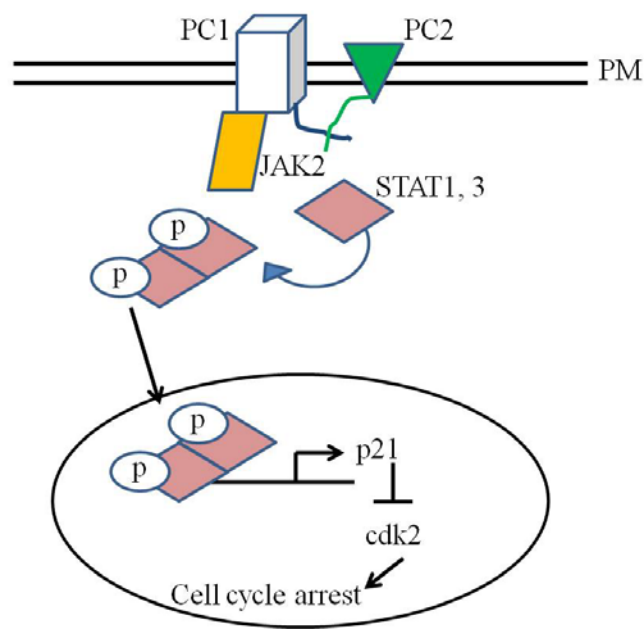
Non-canonical Wnt signaling, or the planar cell polarity pathway, has been implicated in PKD. Renal tubular cells generally divide parallel to the tubule axis. This oriented cell division (OCD) leads to the lengthening of the tubules. Loss of OCD leads to the division of cells at an angle perpendicular to the tubular axis leading to an expansion of tubule diameter rather than its elongation. Defects in OCD have been reported in several mouse models of PKD (56, 57). Additionally, Inversin, a protein product of the gene mutated in nephronophthisis type II (a disease characterized by renal cysts) was shown to act as a switch between canonical and non-canonical Wnt signaling (58).

These results suggest that the dysregulation of canonical and non-canonical Wnt signaling contribute to the pathogenesis of PKD.





**G**





**Figure 1-4: Models for the functions of polycystins.** A: PC1 and PC2 are found on the plasma membrane (PM) in this model. Activation of PC1 leads to the activation of PC2 which leads to calcium influx and changes in gene transcription. B: Polycystins signaling through the primary cilia. Renal cilia bend in response to fluid flow. Bending of the cilia leads to the influx of calcium through polycystins. C and D: Polycystins regulating G-protein signaling. Binding of PC1 to heterotrimeric G proteins activates the  $G\alpha$  subunit and the release of the  $G\beta\gamma$  subunits. This leads to the activation of adenylyl cyclases and MAP kinases which can affect several cellular processes such as cell proliferation, fluid secretion and differentiation. G protein signaling through polycystins can also affect the JNK/AP-1 and NFAT/AP-1 pathways. E: Polycystins signaling through the JAK-STAT signaling pathway. Membrane anchored PC1 binds to JAK2 and activates it which then phosphorylates STAT3 resulting in its translocation to the nucleus. F: PC1 C tail can translocate to the nucleus and coactivate STAT1, 3 and 6 which were already activated independently through cytokine signaling. Once inside the nucleus, STAT transcription factors affect the expression of genes required for cell proliferation, cell growth, differentiation and apoptosis. G: Polycystins regulating the cell cycle through the JAK-STAT signaling pathway. PC1, in a reaction requiring PC2, activates JAK2 which leads to the phosphorylation of STAT1. Phosphorylated STAT1 translocates to the nucleus and activates the cyclin-dependent kinase inhibitor, p21. Activation of p21 results in the subsequent inhibition of cdk2 and cell cycle arrest. Redrawn from references 2 and 131.

## How do cysts form in ADPKD?

Cysts form as small dilations in renal tubules which then expand to form fluid-filled cavities of different sizes. Factors that are thought to lead to cystogenesis include a germ-line mutation in one of the polycystin gene alleles, a somatic second hit which leads to the loss of the normal allele and a third hit which can be anything that triggers the cell to proliferate more leading to the dilation of the tubules. Continued dilation of the tubules through increased cell proliferation, fluid secretion and separation from the parental tubule will lead to the formation of cysts. Other factors that are involved in cystogenesis and/or cyst progression include defective planar cell polarity, extracellular matrix abnormalities, inflammation, increased apoptosis, modifying genes and environmental factors (2, 36).

There is controversy in the PKD field about some factors which are involved in the pathogenesis of PKD such as increased cell proliferation and defective planar polarity. The debate is whether they cause cystogenesis or just assist in making the cysts bigger thereby leading to disease progression. Recently, defective planar cell polarity was ruled out as an initiating mechanism for cystogenesis (discussed in detail below). Germ-line mutations that cause ADPKD are discussed in detail in a previous section. Other mechanisms which cause and/or promote cystogenesis are discussed below.

#### The two-hit or second-hit model for cystogenesis

Microdissection studies on ADPKD kidneys revealed a focal nature for cyst formation in patients. Even though each human kidney is formed of a million nephrons, only a few nephrons (~1000) form cysts in ADPKD patients. All the cells including renal tubular cells in ADPKD patients carry a germ-line mutation in one of the alleles of the polycystin genes. If the germ-line mutation itself is sufficient to cause cystogenesis, all these cells should form cysts. However, as mentioned above, cysts form only in a fraction of these cells. A second-hit theory was proposed to explain this phenomenon. This theory states that in addition to the germ-line mutation in one of the *PKD1* or *PKD2* alleles, a somatic mutation must occur in the other normal allele leading to the complete loss of function of the polycystins causing cyst formation. Cultured epithelial cells from the cysts of ADPKD patients showed loss of heterozygosity in two closely linked polymorphic markers located within the *PKD1* gene in a subset of cysts. Further genetic analysis also confirmed the loss of the normal haplotype in this subset of cysts, thus favoring the second hit hypothesis (59).

## Insights from mouse models

A number of animal models have been described for ADPKD. A *Pkd1*<sup>-/-</sup> knockout mouse model was developed by homologous recombination leading to a frameshift mutation in exon 33 of the *Pkd1* gene. These mice die embryonically. Kidneys in the *Pkd1*<sup>-/-</sup> homozygous mice developed normally until E14.5. Cystic dilations were noted in the proximal tubules at E15.5 which was followed by cysts crowding the entire medulla and most of the cortex in mice which survived to term. These results are consistent with the expression pattern of murine PC1 which peaks at E15.5. This shows the probable role of polycystins in tubular elongation and maintenance of tubular architecture rather than in nephron formation. Pancreatic duct dilations and hypoplastic lungs were some extrarenal manifestation seen in the *Pkd1*<sup>-/-</sup> mice (60).

A *Pkd2*<sup>-/-</sup> knockout mouse model was also developed by targeting mutations into the murine *Pkd2* locus. Similar to the *Pkd1*<sup>-/-</sup> knockout mice, the *Pkd2*<sup>-/-</sup> also showed normal kidney development until E14.5. Cysts began to form in the maturing kidneys by E15.5. Most of these mice died embryonically beginning at E16.5. These results suggested that similar to PC1, PC2 is not required for nephrogenesis but required for the proper maintenance and subsequent elongation of the nephron segments. Extrarenal manifestations such as cardiac defects and pancreatic duct dilations were also seen in these mice (61).

*Pkd1* and *Pkd2* knockout mouse models show that the loss of both alleles of either of these genes is sufficient to cause PKD, hence validating the two-hit hypothesis in animal studies. However, results from *Pkd1* transgenic mice which developed PKD in lieu of persistent expression of *Pkd1*, argue against the second hit theory (62, 63). Another mouse model, expressing a hypomorphic *Pkd1* gene also developed PKD (64). These experimental evidences

suggest that the dysregulation of polycystins leads to cystogenesis rather than its loss or overexpression per se.

#### A critical window for cystogenesis

A study by Piontek *et al* (65) used a tamoxifen inducible *Pkd1* mouse model which shed some light into the mechanism of cystogenesis. They inactivated *Pkd1* in these mice at different time points during and after renal development. When they deleted *Pkd1* in these mice at postnatal day 2 (P2) at a time when cortical nephrogenesis was still ongoing, these mice developed severely cystic kidneys within 2 weeks. They noted that the renal cortex in these cystic mice did not have any dysplastic or immature structures emphasizing the point that *Pkd1* is not required for the initial stages of nephrogenesis. However, when they inactivated *Pkd1* in mice at 3 and 6 weeks of age, these mice did not develop PKD until approximately 5 to 6 months of age. In order to dissect out this time dependent phenotypic difference in cystogenesis, they inactivated the *Pkd1* gene at several time points between P2 and P21. Mice which had *Pkd1* inactivated at P12 or before developed severe PKD within 3 weeks whereas inactivation between P14 and P21 induced a late cystic phenotype at 6 months of age. They observed an increase in cell proliferation rates in early stages (P12-P14) in normal control kidneys compared to normal controls at later stage (P14-P16). A microarray analysis of normal kidneys at ages between P11 and P15 revealed that there is a differential change in gene expression between P11-P12 and between P14-P15. Most of the genes that were differentially expressed were clustered into transporter and catalytic functions. These results suggest that the rate of cyst development in these animals depends upon the developmental state of the kidneys at the time point when *Pkd1* is inactivated. These dramatic differences in phenotype between the inactivation of *Pkd1* in

younger versus older mice also suggest that different pathways may be altered at these different time points leading to rapid cystogenesis earlier and slower cystogenesis later (65).

A recent study by Grantham *et al* followed the diameter, volume and growth rates of cysts in eight ADPKD patients using CT and MR imaging methods for 3 years. All of these patients had normal renal function as measured by glomerular filtration rate. The rate of individual cyst growth in these patients ranged between -2.2 to 71.1% per year. Detection threshold for ultrasound imaging is 7 to 10mm whereas for CT and MR imaging methods it is 2mm. Cysts as big as 10mm in diameter or larger have been detected in ADPKD children who are as young as 1 year old. Most of the cysts in ADPKD begin *in utero* in collecting ducts and an adult collecting duct varies in diameter from 40 $\mu$ m to 100 $\mu$ m as demonstrated by microdissection studies. Fetal collecting ducts are probably smaller than this. With this evidence in hand, Grantham *et al* questioned how fast the cysts should grow *in utero* in order to reach a size detectable by the imaging techniques (2-10mm) at birth or shortly thereafter. For a tubule as big as 100 $\mu$ m in diameter, the growth rate should be 20% per year to grow into a cyst of 2mm in diameter. Grantham *et al* reasoned that even though these numbers can be justified in an adult patient, this would not account for those 2mm cysts that can be detected in ADPKD children. This suggests the possibility that renal cysts that are formed in fetal life grow at a much faster rate in order to reach the detection threshold of the imaging techniques (18).

The mouse model in which *Pkd1* was inactivated between P2 and P12 and developed a rapid cystic disease show that cystogenesis and growth are sensitive to the developmental factors that dissipate approximately 2 weeks after birth in mice. Inactivation of the *Pkd1* gene after this critical time window results in PKD which progress at a much slower pace. In mice, nephrogenesis and tubular elongation continues until 2 to 3 weeks of age which corresponds to

the window of time where they develop rapid cystic disease. In humans, on the other hand, nephrogenesis ceases at approximately 34 weeks of gestation. Renal cysts that are detectable by imaging techniques at birth or in young children with ADPKD may have grown exuberantly in the conducive fetal environment and thereafter, cyst expansion proceeds at a slower rate (18, 65).

### Third hit

A more recent hypothesis has emerged in the ADPKD field, namely, the third hit for cystogenesis. This hypothesis is built upon the second hit hypothesis. The third hit theory states that a cell may not be cystogenic just because it received the first hit (germ-line mutation), and the second hit (somatic inactivation of the normal allele), but that it also requires a third hit which then leads to cell proliferation and cyst growth. The study by Piontek *et al* showed that late inactivation of the *Pkd1* gene in adult kidneys resulted in a slow onset of cystogenesis in mice (65). Lantinga-van Leeuwen *et al* and Takakura *et al* also generated inducible Pkd1 mouse models in which they inactivated the *Pkd1* gene in mature mouse kidneys (66, 67). Takakura *et al* reasoned that the slow onset of PKD when the *Pkd1* gene was inactivated in adult animals could not be justified by the second hit hypothesis and proposed a third hit hypothesis. They stated that if the second hit was enough for cystogenesis, animals which had either the inactivation of both the germ-line *Pkd1* alleles or the transheterozygous mice which had one germ-line *Pkd1* mutation and loss of the other allele by inducible inactivation, should not take the length of time to develop cysts as was seen in these animals. The same group showed later that renal injury resulting from unilateral ischemia reperfusion injury accelerated PKD in the adult inducible Pkd1 knockout mouse model (68). Nephrotoxic injury and ischemia have been described as important stress events that cause a third hit (69, 70).

A similar phenotype as seen in the inducible Pkd1 knockout models was observed when genes required for primary cilia formation were inactivated in developing or adult kidneys. Patel *et al* described a study using an inducible mouse model to knockout the *Kif3a* gene, which is required for cilia formation. While *Kif3a* inactivation and loss of cilia at P2 led to PKD, inactivation of *Kif3a* at P10, P14 or P21 did not result PKD when followed 2 months after gene inactivation (57). The inducible knockout of another ciliary gene *Ifi88* in adult mouse kidneys also led to slow onset cystic disease. More recently, another third hit, unilateral nephrectomy, was added to the list by Bell *et al*. Unilateral nephrectomy leads to the hypertrophy of the remaining kidney and an increase in glomerular filtration rate as it compensates for the loss of the other kidney. As described above, mice which had lost renal cilia (as a result of the deletion of *Ifi88*) in the adult kidney did not develop cystic kidneys until after 6 months. Bell *et al* inactivated *Ifi88* in mouse kidneys at 8 weeks of age, performed unilateral nephrectomy 1 week later and found that within 3 months they developed severe cystic kidney disease in their contralateral kidney. This was associated with hypertrophy which is a normal response when unilateral nephrectomy is performed. Activation of the mTOR pathway was also observed in these mice, as previously shown in association with unilateral nephrectomy-induced hypertrophy. Activation of mTOR pathway in these mice also led to increased cell proliferation which may have resulted in the rapid onset of cystic disease in these mice (69).

#### Cell proliferation and fluid secretion

Epithelial cell proliferation and fluid secretion are two hallmark features of ADPKD (71). Even the gross inspection of polycystic kidneys show increased epithelial cell proliferation evidenced by the increase in the circumference of the kidney tubules that form cysts. Renal tubules normally fall within a range of a few micrometers in diameter. However, in ADPKD,

renal tubules considerably increase in size so as to fall in a size range from millimeters to centimeters when they become cysts. Microscopic examination of polycystic kidneys shows the presence of large numbers of cells in the cyst wall epithelium (72). Several growth-stimulating factors such as the epidermal growth factor (EGF), the transforming growth factor  $\alpha$  (TGF  $\alpha$ ) and the EGF receptor (EGFR) promote cell proliferation in PKD. Overexpression and mislocalization of EGFR to the apical side of the cystic epithelium has been reported in several animal models and in human PKD patients. Transgenic animals overexpressing TGF  $\alpha$  also develop PKD (11).

An important factor which leads to the proliferative phenotype in ADPKD is the second messenger, adenosine 3', 5' cyclic monophosphate (cAMP). cAMP is an intracellular mediator of adenylyl cyclase signaling and is shown to stimulate proliferation of cyst-lining cells in human PKD. The mammalian kidney is a target of several hormones such as arginine vasopressin (AVP), parathyroid hormone, secretin and vasoactive intestinal peptide as well as autocooids such as prostaglandins and adenosine, all of which lead to the elevation of the intracellular concentration of cAMP. In normal human kidneys, cAMP stimulation leads to a non-mitogenic response. However, ADPKD kidneys show a proliferative response to cAMP stimulation. cAMP mediates its effect in PKD by activating the B-Raf/MEK/ERK pathway. Moreover, normal human kidney (NHK) cells behave like ADPKD cells with a cAMP stimulatory phenotype when treated with calcium channel blockers. In contrast, the mitogenic response to cAMP was reversed in ADPKD cells when they were treated with calcium channel activators or calcium ionophores (16, 44, 73).

During the early stages of cystogenesis, cysts are attached to their parental renal tubules and a derivative of the glomerular filtrate enters the cysts. Once these cysts expand to approximately 2mm in diameter, the cyst closes off from its parental tubule and now fluid can



enter the cysts only through transepithelial secretion. A transporter present in the basolateral membrane of cyst-lining cells, the Na-K-Cl-transporter, mediates the entry of chloride into the cytoplasm. Further entry of chloride into the cyst lumen is mediated by a chloride transporter present on the apical membrane of cystic epithelial cells, cystic fibrosis transmembrane conductance regulator (CFTR) (1, 11). The activity of CFTR increases in ADPKD possibly due to secondary effects from the increased concentration of cAMP (1). It has also been shown that patients who have cystic fibrosis and PKD have a milder disease (74).

#### Other molecular abnormalities in ADPKD

An increase in apoptotic DNA fragmentation and in-situ labeling of apoptotic cells was described in PKD patients and in two mouse models of PKD. Apoptotic cells were present in both cystic and non-cystic tubules as well as in glomeruli. The progressive loss of renal function seen in PKD may be explained by this increase in apoptosis. Homozygous deletion of the anti-apoptotic gene, *Bcl-2*, results in PKD in mice (7). ADPKD tissues also show an increase in the expression of c-myc. Moreover, kidney specific overexpression of c-myc in mice results in renal cysts characterized by both an increase in proliferation and apoptosis (11).

Abnormalities in the innate immune system are another hallmark feature in both ADPKD and ARPKD. In ADPKD, accelerated production of monocyte chemotactic protein-1 (MCP-1) leads to an increase in mononuclear cells in the renal parenchyma. Abnormal urinary excretion of MCP-1 in early stage ADPKD patients shows that the immune system pathway is dysregulated during early stages of the disease. Analysis of the *cpk* mouse model which resembles human ARPKD, showed that around 60 monocyte/macrophage markers were overexpressed in the *cpk* mice. Genome wide transcription profiling in the *cpk* mouse model and

a rat model of PKD, Han:SPRD, showed an overexpression of macrophage markers and other innate immune factors. The expression pattern of one of these overexpressed macrophage markers, CD14, was analyzed in cpk mice, ADPKD and ARPKD patients. In the cpk mice, CD14 expression increased with increasing age in the controls and there was a significant increase in cystic kidneys compared to age-matched littermate controls. However, the number of CD14 positive mononuclear cells could not account for this increase in CD14 expression. Further histological analysis confirmed staining of CD14 in the proximal tubules and principal cells of collecting ducts and in cysts derived from these segments. Although CD14 is a membrane anchored receptor, once activated, soluble CD14 can be shed from the membrane. Both ARPKD and ADPKD patients showed an abnormal increase in the shedding of CD14. In ADPKD kidneys, these shed soluble forms of CD14 were washed out into the urine (75).

CD14 is a potent stimulator of TNF $\alpha$  secretion which can lead to a cystogenic pathway. Shed forms of CD14 are also mediators for renal endotoxin-induced tubulointerstitial injury. An injury related CD14 overexpression is also seen in renal ischemia reperfusion injury animal models. CD14 is a major ligand for Toll-like receptor-4 (TLR4), through which it may transactivate cystogenic pathways such as signaling through Wnt, AP-1 and PI3K pathways (75).

PC1 has been shown to be associated with the extracellular matrix (ECM) components and is thought to have a role in cell-extracellular matrix interactions. Owing to this role of PC1, alterations in the tubular basement membrane (TBM) including its thickening, splitting, fraying and multilayering have been seen in human PKD. Cystic kidneys from several animal models of PKD also show BM abnormalities including thickened and laminated basement membranes as well as increased expression of  $\alpha$ 1 type IV collagen and laminins  $\beta$ 1 and  $\beta$ 2 (76). A hypomorphic laminin  $\alpha$ 5 mouse model has also been shown to develop PKD (77). Speculation of PC1 working

as a receptor for extracellular matrix molecules and its expression in the basolateral surfaces may explain the PKD phenotype seen in the hypomorphic laminin  $\alpha 5$  mutant. Moreover, recombinant PC1 peptides have also been shown to bind laminin *in vitro*. Increased EGF expression, an abnormality observed in PKD, may also be indirectly involved in modulating the effects of laminin since many of the laminin chains consist of multiple EGF-like repeats some of which even bind to the EGF receptor and signals downstream via this pathway (77).

As mentioned in a previous section, polycystins by virtue of their location on the primary cilia of renal epithelial cells are thought to play a role in mechanosensation. In response to fluid flow and bending of the cilia, polycystins present on the cilia mediate the entry of calcium into cells. *Pkd1*<sup>-/-</sup> renal epithelial cells, have normal primary cilia, however, they lack the flow-induced calcium response of normal cells (43). Mutations in any of the polycystins as it occurs in ADPKD, leads to abnormalities in the fluid-flow sensing mechanism which may contribute to cyst development. A kidney specific inactivation of *Kif3a*, a subunit of kinesin-II that is essential for cilia formation, inhibited renal ciliogenesis and resulted in a subsequent development of PKD in mice (78).

Defective planar cell polarity is another hallmark feature seen in PKD. This phenotype can also be attributed to the presence of cystoproteins in cilia. It has been proposed that cilia in the developing kidney are responsible for the oriented cell division (OCD) along the length of a renal tubule. Loss of OCD was observed during advanced stages of cystogenesis in the *pck* rat, an orthologous model of ARPKD. Measurement of mitotic angles in the *pck* rat showed that the loss of OCD preceded tubular dilation in these animals. Kidney specific inactivation of *HNF1 $\beta$* , a transcription factor involved in the regulation of expression of *Pkd2* and *Pkhd1*, also resulted in PKD as a result of severe mitotic angle distortion and loss of OCD seen even at birth (56).

However, recent results with the kidney specific inactivation of *Pkd1*, *Pkd2* and *Pkhd1* mouse models show that loss of OCD is not required for cystogenesis. Precystic tubules from conditional *Pkd1* or *Pkd2* mice did not show the loss of OCD. Loss of OCD was observed once the tubules dilated to form cysts. The conditional deletion of *Pkhd1* specifically in the kidneys resulted in the loss of OCD, however, these mice did not develop PKD (79).

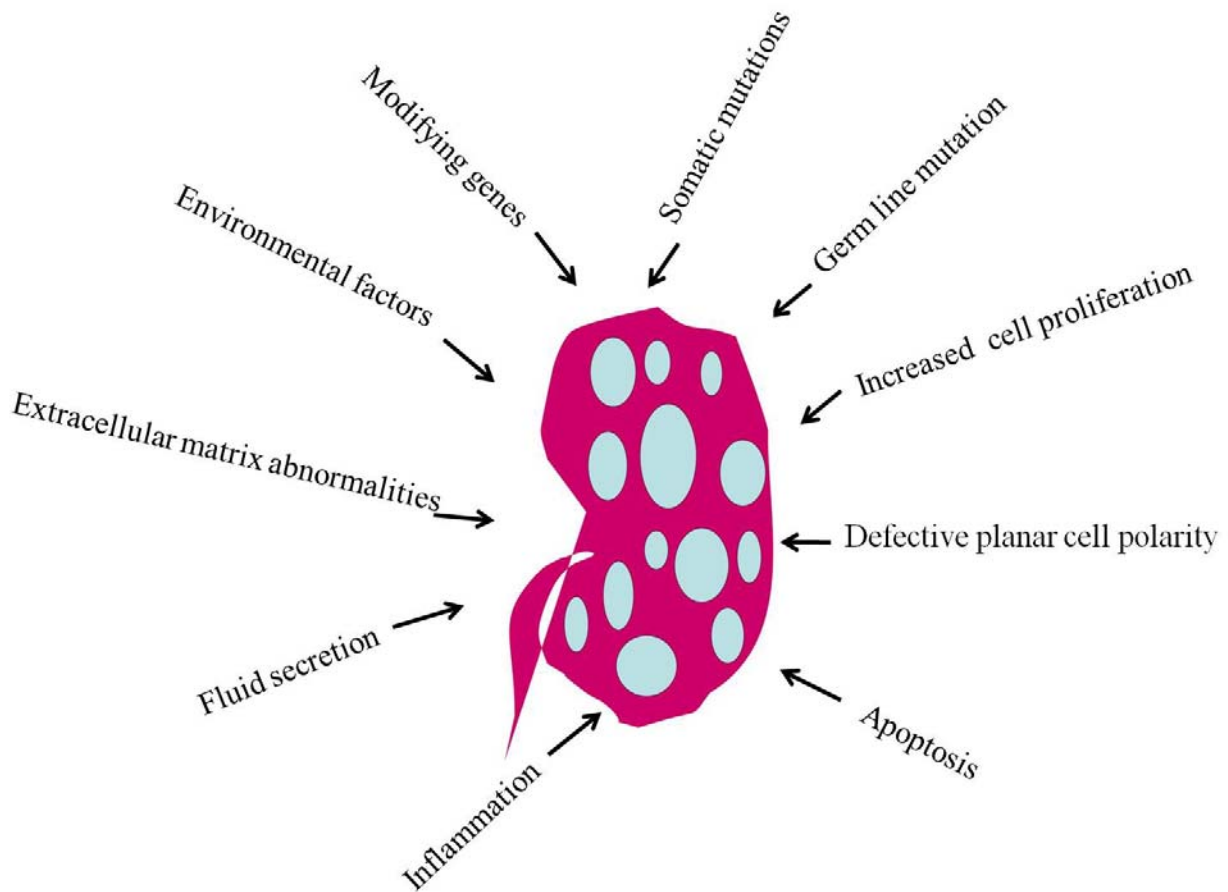
ADPKD is a fully penetrant trait. Virtually 100% of all individuals who inherit one of the mutated polycystin genes will develop PKD in their lifetime. The disease presentation and severity, however, vary among ADPKD patients, even within family members (2). This phenotypic variability associated with disease presentation led to the investigation of modifying loci and environmental factors that may influence disease progression. Although ADPKD is a single-gene disorder, the disease phenotype is a complex trait. In single-gene disorders with complex traits, the primary mutant gene product is embedded in a complex system that includes genetic modifiers and modulating environmental factors (80).

Belibi *et al* tested the effects of caffeine on normal and ADPKD cultured cells. Caffeine inhibits phosphodiesterases (PDE), the enzymes which hydrolyze cAMP thereby leading to the accumulation of this second messenger. As mentioned previously, increased cAMP levels can result in a mitotic response in the ADPKD cells. This study found that caffeine and other PDE inhibitors such IBMX and rolipram increased intracellular cAMP levels in ADPKD cells (81). Therefore, caffeine is an important environmental influence in the progression of ADPKD which patients should avoid.

Early onset of PKD is seen in patients with microdeletions in the *PKD1* and the *TSC2* genes which are present on the same chromosome adjacent to each other (82, 83). Bilineal

inheritance of heterozygous *PKD1* and *PKD2* mutations also lead to severe renal disease in some patients (83). Transheterozygous mutations in the *Pkd1* and *Pkd2* genes in mice has also been reported to have a greater than additive renal disease phenotype. Moreover, patients who have cystic fibrosis and PKD have a milder disease (74).

The possible existence of modifier genes in ADPKD patients was also confirmed from an analysis carried out in monozygotic (MZ) twin siblings. Large intrafamilial variability was observed in the progression of renal disease in these MZ twins (84).



**Figure 1-5: Various mechanisms of cystogenesis and disease progression in ADPKD**

## Genes that modify PKD phenotype

*Pax2* is a developmentally regulated gene which is required for the differentiation and proliferation of the renal epithelium in both mice and humans. Expression of *Pax2* is seen in the nephric duct, ureteric bud and induced mesenchyme in the normal developing kidney. *Pax2* is expressed in the nephrogenic zone of the kidney from E11 to postnatal day 10 in mice (85). *Pax2* null mice lacked the urogenital system including absent kidneys and ureters whereas overexpression of *Pax2* led to multicystic disease (86). Human juvenile cystic kidneys, ADPKD kidneys and *cpk* (a mouse model which resembles ARPKD) mouse kidneys show persistent expression of *Pax2* (85).

Expression of *Pax2* during kidney development and in cystic kidneys prompted two independent groups of investigators to ask the question if *Pax2* is involved in cystogenesis. Ostrom *et al* showed that reduced gene dosage of *Pax2* can modify the cystic phenotype in the *cpk* mouse model of PKD. Mice homozygous for the *cpk* mutation and heterozygous for the *Pax2* mutation had reduced cystic severity and a longer life span (85).

The role of *Pax2* in cystogenesis was also investigated in the *Pkd1* null mice. Fetal kidneys (E18.5) from the *Pkd1* null mice showed the presence of *Pax2* in the cystic epithelium, bifurcating ureteric buds, and also in the condensing mesenchyme. Double mutant mice which were *Pkd1* null and heterozygous for the *Pax2* mutation (*Pkd1*<sup>-/-</sup>;*Pax2*<sup>+/-</sup>), showed a marked reduction in kidney mass and cyst size when compared to *Pkd1*<sup>-/-</sup>;*Pax2*<sup>+/+</sup> mice (86).

The role of another developmentally regulated gene, *Cux1*, was also investigated in PKD. *Cux1* is a homeobox gene important during renal development. *Cux1* is also ectopically

expressed in several mouse models of PKD as well as in human ADPKD kidneys. A mutation in *Cux1* modified cyst progression in the *cpk* mouse model of PKD (87).

## The Cut family of proteins

*Cux1* (Cut homeobox 1) is a murine homologue of the *Cut* homeobox gene in *Drosophila melanogaster* (88). Homeobox genes encode homeodomains which are 61 amino acid long DNA binding domains. The homeodomain was the first protein domain that was discovered in *Drosophila* embryonic development and later through sequence comparison found to be conserved among higher eukaryotes. Mutations in the homeobox genes in *Drosophila* result in “homeotic transformations”. Homeotic transformation is a developmental anomaly where one body part transforms into and acquires the characteristics of another body part (89, 90).

Several mutations have been described in the *Drosophila Cut* locus. Embryonic lethal mutations in the *Cut* locus fall into two complementation groups, lethal I and lethal II. These mutations result in the transformation of the external sensory (es) organs into internal sensory (chordotonal, ch) organs (90-92). Viable mutations in the *Cut* locus result in morphological changes in the wings (cut wing, ct) or the legs (kinked femur, kf) (93). The locus was named *Cut* after the “cut wing” phenotype.

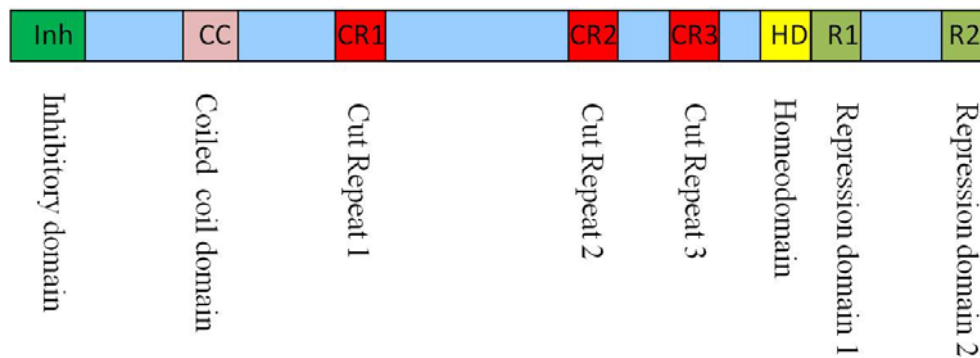
Cut proteins are expressed in the insect excretory organs, Malpighian tubules. Malpighian tubules serve as primitive kidneys in *Drosophila*. Some mutations in the *Cut* locus result in the transformation of Malpighian tubules into gut tissue showing that the Cut proteins are required for the proper development of Malpighian tubules (94).

A human homologue of Cut, CDP (CCAAT displacement protein) or CUTL1 (Cut-like 1), was first purified from HeLa cells (95, 96). As the name implies, CDP acts as a

transcriptional repressor by preventing the binding of positively acting CCAAT transcription factors to promoters (95). The human *CUTL1* gene is a candidate tumor suppressor gene present on chromosome 7q22. This region is frequently rearranged or deleted in uterine leiomyomas, acute myeloid leukemia and myelodysplastic syndrome. Other mammalian homologues of *Cut* include *Clox* (dog) and *CDP2* (rat) (88). Another *Cut* family member, *Cux2*, has also been described in mice. *Cux2* is exclusively expressed in the nervous system (96). The Human Genome Organization (HUGO) has recently changed the nomenclature of *Cut* genes and proteins to *Cux1* (gene) and *Cux1* (protein) to describe the mouse counterparts and *CUX1* (gene) and *CUX1* (protein) to describe the human homologues (97).

There are five evolutionarily conserved domains in the *Cut* proteins. These include a region which will form a coiled-coil structure, three *Cut* repeats and the *Cut*-Homeodomain (HD) (98). The homeodomain is encoded by the homeobox found on the C- terminal region of the protein. The *Cut* repeats (CR) are stretches of 60-70 amino acids which have 55-68% amino acid identity and are known as CR1, CR2 and CR3. *Cut* repeats also have 59-64% nucleotide identity suggesting their origin from a duplication event (90, 93, 98). Murine and human homologues of *Cut* proteins have two repression domains located at the carboxy-terminal region (99, 100). These repression domains in human *Cut* proteins map to two subdomains of 81 and 29 amino acids. They independently repress gene expression from activated promoters (Figure 1-6) (100).





**Figure 1-6 Structure of Cut proteins.** The N-terminal domain of Cux1 has an inhibitory domain whereas the C-terminus has two repression domains. These proteins also have a coiled coil domain, three Cut repeats and a homeodomain. Redrawn from reference 97.

Cut proteins are believed to function as transcription factors in flies and mammals. Accordingly, they have been localized to the nucleus in both species (100). In mammals, Cut proteins were primarily characterized as transcriptional repressors. However, recent evidence indicates that Cux1 can act as transcriptional repressors or activators. Cux1 is capable of binding to a wide range of DNA sequences including CCAAT, ATCGAT, Sp1 sites and AT-rich matrix attachment regions. Evaluation of the DNA binding mechanism of Cut proteins revealed that Cut repeats cannot bind DNA as monomers. However, combinations of domains such as CR1CR2, CR3HD, CR1HD and CR2HD exhibit high DNA binding affinities. The CR1CR2 dimer showed rapid on-off rates and hence transient DNA binding, while CR3HD showed slow and stable DNA binding. The CCAAT displacement activity of Cut proteins involve the CR1CR2 domains and the homeodomain is not involved in this process (98).

Transcriptional repression by Cux1 is mediated by two different mechanisms. The first mechanism involves passive repression where these proteins compete with activators such as Sp1 or CCAAT -binding factor for binding sites on the DNA. The second mechanism involves active repression where Cux1 binds to DNA with its repression domains at a large distance from the

transcription start site of the gene. Cux1 binds to DNA in this manner through DNA looping (99, 100).

The transcriptional repression targets of Cut homologues include *γ-globin*, *c-Myc*, *myosin heavy chain*, *NCAM* and *CD8a*, *c-mos*, *MMTV long terminal repeats*, *gp91-phox*, *HNP*, *C/EBP epsilon*, *lactoferrin* *Rnf35*, *p21* and *p27* genes (97, 101).

## Regulation of Cux1

### Transcriptional regulation of Cux1

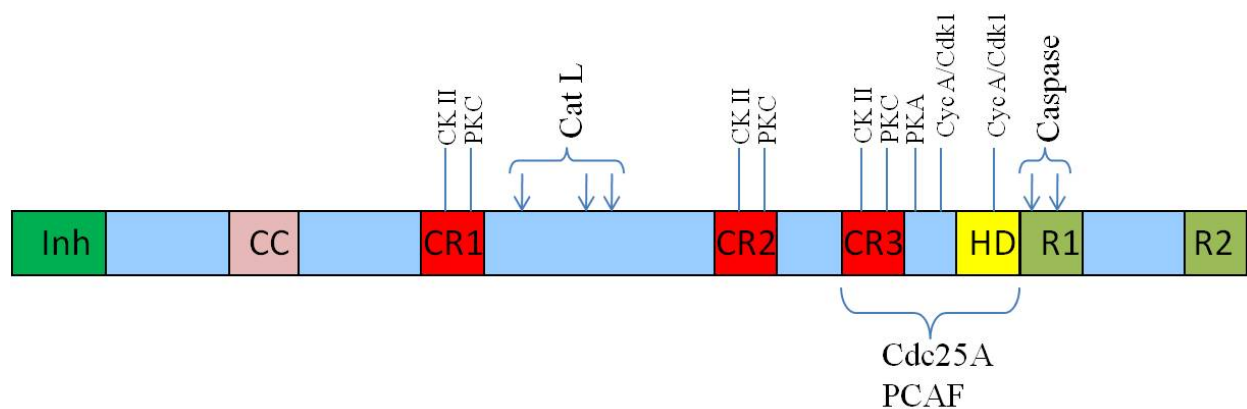
Regulation of *Cut* by the Notch and Wingless signaling pathways have been described for cell-type specification during *Drosophila* wing development (99). Downregulation of *Cut* by Notch was also shown in *Drosophila* oogenesis (102). During mouse embryonic development, co-expression of Cux1 and Notch signaling pathway components is seen in multiple tissues. Most of these tissues co-expressed Cux1 with the Notch receptors 1 or 3 and the Notch pathway ligand Jagged1. In the developing kidney, Cux1 is co-expressed with Notch pathway components in the condensing mesenchyme, comma and S-shaped bodies and in the presumptive podocytes of capillary loop stage glomeruli. Cux1 was also significantly upregulated in a rat kidney epithelial cell line, RKE which constitutively expressed Notch1. This increase in Cux1 expression correlated with the decrease in the expression of a cyclin dependent kinase inhibitor (CKI) p27, a transcriptional repression target of Cux1 (103). A direct interaction between Cux1 and Grg4 (Groucho 4), an effector of Notch signaling pathway has also been shown (104).

*CUX1* was also shown to be a transcriptional target of transforming growth factor  $\beta$  (TGF $\beta$ ), a major modulator of tumor cell migration. In various tumor cell lines, CUX1 activity was associated with an increase in migration and invasiveness both *in vivo* and *in vitro*. CUX1

was identified as the mediator of pro-migratory effects of TGF $\beta$ . Transcriptional upregulation of CUX1 by TGF $\beta$  leads to the activation of a transcriptional program regulating genes involved in cell motility, invasion and extracellular matrix composition (105).

#### Post-translational regulation of Cux1

Post-translational modifications of Cux1 include phosphorylation, dephosphorylation, proteolytic processing and acetylation (Figure 1-7) (97). Both Casein kinase II and protein kinase C (PKC) phosphorylate Cux1 at serine/ threonine residues within the cut repeats (97, 106, 107). Protein kinase A (PKA) phosphorylates Cux1 at a serine in the linker region between CR3 and HD (97, 108). Cyclin A/ Cdk2, the principal cyclin-cdk complex during S phase, interacts with Cux1, however, Cux1 is not phosphorylated by this complex. As the cell moves out of S phase into G2 phase, Cyclin A/ Cdk1 becomes the main cyclin-cdk complex which phosphorylates Cux1 in the linker region between CR3 and HD and also within the homeodomain (97, 109).



**Figure 1-7 Post-translational modifications of Cux1.** Casein Kinase II (CKII), Protein Kinase C (PKC) and Protein Kinase A (PKA) phosphorylate Cux1 at different sites. CKII and PKC phosphorylate Cux1 in all three Cut repeats. CyclinA/Cdk1 complex also phosphorylate Cux1 in the linker region between CR3 and HD and also in the HD. Cux1 is dephosphorylated by Cdc25A and acetylated by PCAF in the CR3HD region. Cleavage sites for the nuclear form of Cathepsin-L is found in the linker region between CR1 and CR2. Caspases also proteolytically process Cux1 and two caspase cleavage sites are found on the first repression domain. Redrawn from reference 97.

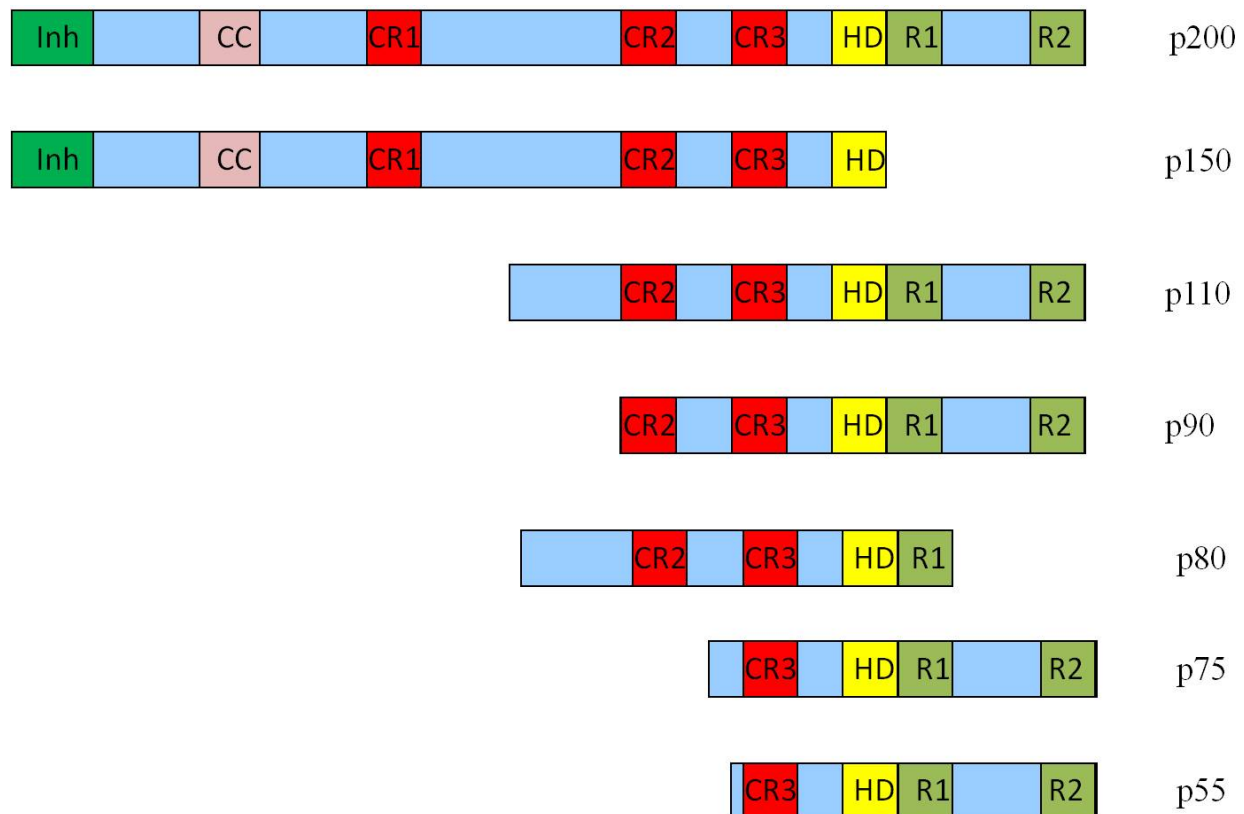
Acetylation occurs at conserved lysine residues around the HD region of Cux1, mediated by PCAF acetyl-transferase. Cdc25A phosphatase dephosphorylates Cux1 at the CR3HD region which leads to an increase in DNA binding at G1/S transition. Acetylation and phosphorylation events negatively regulate Cux1 by causing an inhibition in DNA binding and transcriptional regulation. In contrast, dephosphorylation events lead to an increase in DNA binding (97).

Proteolytic processing of Cux1 is carried out by a nuclear form of cathepsin-L and caspases which generate various short isoforms of Cux1 (Figure 1-8) (97).

### Cux1 Isoforms

Cux1 isoforms exhibit distinct DNA binding and transcriptional properties. These isoforms are known by their apparent molecular weights. Cux1 exists in its full length form, p200, during early G1 phase. Full length Cux1 cannot stably bind to its consensus target sequence. As the cell progresses into S phase, Cux1 gets processed into a shorter isoform p110, that can stably bind DNA (110). The p110 isoform lacks the N-terminal inhibitory domain and the first Cut repeat (110, 111). Processing of p200 Cux1 to form p110 is carried out by a cysteine protease, cathepsin-L. Cathepsins are lysosomal proteases, however, an isoform of cathepsin-L is localized to the nucleus during the G1/S transition of cell cycle and proteolytically processed the p200 Cux1 to form the 110kDa Cux1 (112). Another shorter Cux1 isoform, p90 is also generated during the G1/S transition by cathepsin-L cleavage (97, 110). A C-terminally truncated Cux1 isoform, p150 is generated which is incapable of binding DNA. It is not known what proteolytic event generates this isoform. The p150 isoform functions in a dominant negative manner in the lactating mammary gland (97, 113). Two processing events mediated by cathepsin-L and caspases generate a p80 isoform of Cux1 (97, 111). A 55kDa isoform of Cux1

is present in mouse testis (114). Transcription initiation within intron 20 of *Cux1* gene generates a shorter isoform of Cux1, p75 which contains only two domains, CR3 and HD. The p75 isoform stably binds DNA and can act as both a transcriptional repressor of p21 and a transcriptional activator of DNA polymerase  $\alpha$  (115).

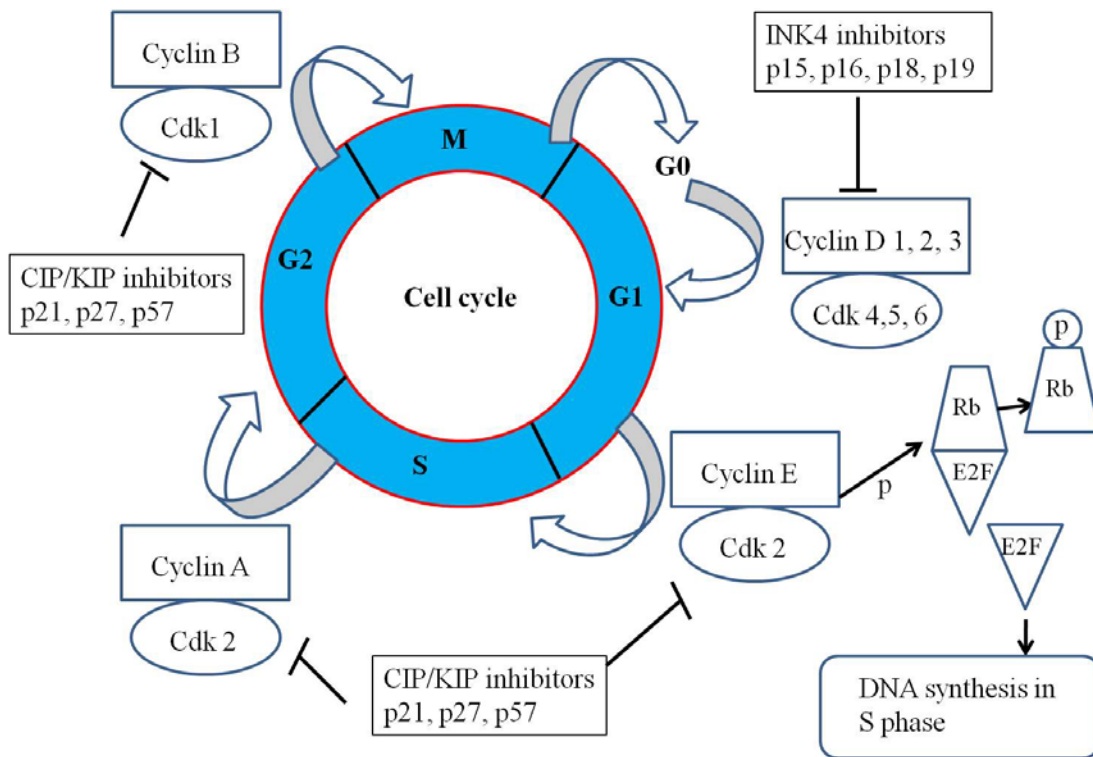


**Figure 1-8 Cux1 isoforms.** Various isoforms of Cux1 are named according to their apparent molecular weights. The full length Cux1 is p200. Proteolytic processing by Cathepsin-L and Caspases generate p150, p110, p90 and p80 isoforms. The p55 isoform is present in the testis. Alternate transcription initiation at a start site in intron 20 generates the p75 isoform. The p200 isoform acts only as a transcriptional repressor whereas the p75, p80, p90 and p110 isoforms can function as repressors or activators depending on the promoter. The p150 form is known to function in a dominant negative manner. Redrawn from reference 97.

## Role of Cux1 in cell cycle regulation

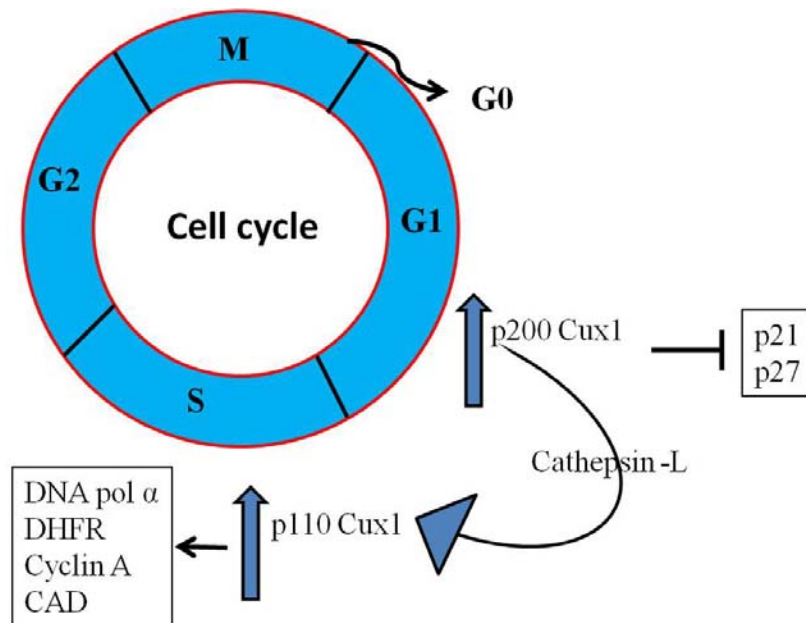
The cell cycle is broadly divided into two stages- interphase and mitotic phase. Mitotic phase involves cell division and is characterized by different stages known as prophase, metaphase, anaphase and telophase. Interphase is the interlude between two mitotic phases. Interphase is divided into 3 different stages known as G<sub>1</sub>, S and G<sub>2</sub> phases. The cell is prepared for DNA synthesis in G<sub>1</sub> phase, DNA replication occurs in S phase and G<sub>2</sub> phase prepares the cell for the upcoming mitosis. Cells in G<sub>1</sub> phase can stay in a resting state known as G<sub>0</sub> before committing to enter into DNA replication (116).

Correct cell division is ensured by tight control of the cell cycle through a variety of mechanisms. Transition of cells from one phase to the next is controlled by a set of different cellular proteins (Figure 1-9). These include cyclins, cyclin dependent kinases (CDK) and cyclin dependent kinase inhibitors (CKI). Specific cyclin-CDK pairs are activated at specific points of the cell cycle (116). The active complex formed between cyclins and CDKs will phosphorylate the retinoblastoma protein (Rb) which is usually bound to the transcription factor E2F. Phosphorylation of Rb releases E2F which in turn activates genes required for DNA synthesis (117). CKIs are cell cycle inhibitory proteins which counteract the activity of CDKs either by binding to a particular CDK or binding to the CDK-cyclin complex. Two distinct families of CKIs are known, the INK4 family and the CIP/KIP family. The INK4 family of inhibitors specifically inactivates the G<sub>1</sub> CDK-cyclin complex and includes p15, p16, p18 and p19. The CIP/KIP family of inhibitors works by inhibiting the G<sub>1</sub> CDK-cyclin complex thereby regulating G<sub>1</sub>-S transition and also by inhibiting the CDK1-cyclinB complex during mitosis to a lesser extent. The CIP/KIP family includes p21, p27 and p57 (116). p21 and p27 are transcriptional repression targets of Cux1 (101, 118).



**Figure 1-9 Cell cycle regulation.** Upon receiving a stimulus to divide, cells enter the cell cycle which is characterized by two main phases- the interphase and the mitotic phase. Interphase is characterized by DNA replication and preparation of the cell to divide into two before the mitotic phase. After mitosis, cells that do not divide further will stay in a quiescent state in the G0 phase. Positive and negative regulation of the cell cycle is shown in the figure which is carried out by the cyclin/ cyclin dependent kinase (CDK) complexes and the cyclin dependent kinase inhibitors (CKI- CIP/KIP and INK4). As shown, there are specific cyclin/CDK complexes and CKIs for different phases. INK4 inhibitors mainly inhibit the G0/G1 transition whereas the CIP/KIP inhibitors are more promiscuous inhibitors.

In *Drosophila*, Cut proteins are known to be involved in cell type specification during cellular differentiation. The role of mammalian Cut proteins in the cell cycle was first shown by their ability to downregulate the CKI, p21 (118). Later it was also shown that Cux1 can repress another CKI, p27, in mammalian kidneys (101).



**Figure 1-10 Cux1 in cell cycle.** Full length p200 Cux1 is highly expressed during the G1 phase. Cdc25A phosphatase that is expressed highly during the G1/S transition dephosphorylates Cux1 and increases its DNA binding. The p200 Cux1 binds DNA transiently and transcriptionally represses the CKIs, p21 and p27. By repressing the expression of these inhibitors, Cux1 drives the cell forward into S phase. Near the end of the G1/S transition, there is an increase in the expression of the nuclear form of Cathepsin-L which cleaves Cux1 to form the p110 isoform which transcriptionally activates the genes required for DNA synthesis in S phase such as DNA polymerase  $\alpha$ , DHFR, Cyclin A and CAD.

The DNA binding activity of mammalian Cut proteins is regulated in different phases of the cell cycle. During G0 and early G1 phases, Cut DNA binding activity is very weak unless the cell extracts were previously treated with alkaline phosphatase. Strong DNA binding is seen in S phase which is associated with the dephosphorylation of Cut proteins by Cdc25A phosphatase. Overexpression of Cdc25A phosphatase triggers the G1-S transition in cell cycle (110, 118). The increase in the activity of Cux1 also results in the downregulation of p21. Cux1 repressed the p21 promoter reporter activity in cotransfection experiments whereas an antisense Cux1 vector



restored p21 expression (110). These events are carried out by the full length p200 Cux1 which rapidly binds DNA (119).

The p110 isoform is generated as cells progress into the S phase of cell cycle by the increase in expression of nuclear cathepsin-L which subsequently cleaves p200 Cux1. Unlike the p200 isoform, p110 can act both as a transcriptional repressor or activator depending on the promoter context (119). Cux1 was previously shown to be the DNA binding subunit of the promoter complex, histone nuclear factor-D (HiNF-D) which interacts with several cell cycle regulatory elements of histone genes during the G1/S transition of cell cycle. Several genes which are upregulated during S phase were found to be transcriptionally activated by p110 Cux1. These genes include DNA polymerase  $\alpha$ , cyclin A, dihydrofolate reductase (DHFR) and carbamoyl-phosphate synthase-aspartate carbamoyltransferase-dihydrorotase (CAD). The full length Cux1 was unable to carry out these transcriptional activation functions. Later, during G2 phase, DNA binding of Cux1 decreases owing to its phosphorylation by cyclin A or Cdk1 within the homeodomain (120).

### Role of Cux1 in Kidney development

Cux1 is expressed in the developing brain, limb, lung and kidney during mouse embryogenesis. High expression of Cux1 can be seen in the metanephric kidney shortly after metanephric induction and it remains high until the completion of nephrogenesis. Cux1 expression decreases after nephrogenesis and adult kidneys show very low levels of Cux1. *In situ* hybridization shows high Cux1 expression in the nephrogenic zone of the kidney in uninduced and condensed mesenchyme and also in developing epithelial structures such as ureteric bud,

renal vesicles and S-shaped bodies. By the end of nephrogenesis, Cux1 is downregulated and only low levels of it can be detected in the mature tubules and glomeruli (101).

Studies using *CMV/Cux1* transgenic mice which overexpressed the *Cux1* gene under the control of CMV promoter revealed the role of *Cux1* in cell cycle and its regulation. The transgenic mice showed multiorgan hyperplasia including kidney hyperplasia. The CKI p27 was found to be downregulated in the developing kidneys of these mice (101). Increased expression of Cux1 in mesangial cells resulted in mesangial cell expansion and cell proliferation. The overexpression of Cux1 also resulted in the development of glomerulosclerosis, interstitial fibrosis and proteinuria (121). *Cux1* transgenic mice developed hepatomegaly which correlated with increased cell proliferation. Ectopic expression of Cux1 was limited to a population of small cells in the transgenic livers but not in mature hepatocytes. Many of these small cells also expressed markers of cell proliferation (117).

Several Cux1 knockout mouse models have been generated. One of these includes a knockout in which Cux1 lacked the first Cut repeat (*Cux1*<sup>tm1Ejn</sup> or *Cux1*ΔCR1). Wavy hair and curly whiskers were observed in these mice. Impaired lactation was observed in homozygous females which resulted in ~50% postnatal lethality in their litters. These mice had phenotypically normal kidneys (97). Another knockout mouse model was generated with a C-terminal deletion of the protein which included the homeodomain and repression domains (*Cux1*ΔHD). The resulting knockout mice have a complex phenotype such as partial neonatal lethality, stunted growth, wavy fur and whiskers and latent alopecia. These mice also showed muscle wasting with loss of body fat similar to cachexia. The complexity in the phenotype can be attributed to the role of Cux1 in the transcriptional regulation of multiple target genes (122). Luong *et al* generated a similar knockout mouse line in which the homeodomain and the C terminus of the Cux1 protein

were genetically deleted (Cux1 $\Delta$ C). This mutation led to decreased immune function, hyperplasia of myeloid cell lines, abnormal formation of the hair fibers and reduced fertility (123). The mutant protein in both Cux1 $\Delta$ HD and Cux1 $\Delta$ C mice localize to the cytoplasm indicating the loss of nuclear localization of Cux1. Therefore, homozygous Cux1 $\Delta$ HD and Cux1 $\Delta$ C mice have a functional loss of Cux1 (122, 123).

## Role of Cux1 in PKD

Cux1 expression was examined in the *cpk* mouse- a mouse model that resembles human ARPKD. Cystogenesis in *cpk* mice starts at E17 and affected mice die of uremia by 3-4 weeks of age. Cux1 was found to be highly expressed in the cyst epithelium in 21 day old mice (late stage cystogenesis) but not in phenotypically normal littermates. The increase in Cux1 resulted only in a slight increase of mitotic activity in the polycystic kidneys of these mice compared to the kidneys of normal mice. However, cyst epithelial cells remained highly undifferentiated. Therefore, the overexpression of Cux1 in these cysts correlated better with the absence of cellular differentiation than increased cellular proliferation (88).

Cux1 expression was also evaluated in a *Pkd1* null mouse model, a model for ADPKD. Cux1 was highly and ectopically expressed in both cystic and normal tubules in the cystic kidneys from early and late stage *Pkd1* null embryos. This also correlated with increased cell proliferation since both Cux1 and PCNA (a cell proliferation marker) co-localized in cells lining the cysts. A decrease in p27 expression was seen during late stages of cystogenesis. In the kidneys of *cpk* mice, Cux1 upregulation was not observed until late stages of cystogenesis. Whereas no p21 was detected in the *Pkd1* null embryo kidneys, Cux1 and p21 were co-localized

in cyst lining cells in the cpk mice. These cystic cells also showed an increased incidence of apoptosis (124).

The levels of the full length, p200 Cux1, were increased in human ADPKD kidneys compared to normal human kidneys. A decrease in the nuclear form of Cathepsin-L was also seen in the ADPKD kidneys. A decrease in Cathepsin-L resulted in a reduction in the proteolytic processing of Cux1, resulting in the accumulation of p200 Cux1 which transcriptionally represses p21 and p27 thereby increasing cell proliferation (87).

The p75 isoform of Cux1 was observed to be overexpressed in *Pkd1*<sup>-/-</sup> polycystic kidneys. Sustained expression of p75 Cux1 in transgenic animals resulted in renal cystogenesis in a dosage-dependent manner. Cysts were observed by 12-18 months of age in these mice. Increased proliferation was observed in cyst lining cells of these mice. The rate of apoptosis was normal. At a molecular level, these mice showed downregulation of p27 and upregulation of c-Myc (125).

## Cux1 as a modifier of the PKD phenotype

An ARPKD mouse model carrying a targeted mutation in the *Cux1* gene was generated to determine the role of Cux1 in the progression of PKD. The cpk mouse model that resembles human ARPKD was crossed to *Cux1*<sup>tm1Ejn</sup>. This *Cux1* mutant mouse carried an in-frame deletion of *Cux1* that spanned exons 15 and 16. The phenotype of *Cux1*<sup>tm1Ejn</sup> included wavy hair and curly vibrissae. Mutant females had a lactation defect and were unable to support pups. The kidneys of these mice were phenotypically normal. The *Cux1*<sup>tm1Ejn</sup> phenotype was similar to the mutations in the EGFR pathway. Genetic or pharmacologic inhibition of EGFR activity had been shown to result in decreased cyst formation and improved kidney function. The *Cux1*<sup>tm1Ejn</sup> mice

were crossed with Cys1<sup>cpk</sup> mice anticipating a similar alteration in cyst progression. The double mutant mice indeed showed a modification in cyst progression. However, the mutation in *Cux1* did not reduce the cyst progression as expected, rather it accelerated the disease progression in these animals. This phenotype was explained by the ectopic expression of Cux1 $\Delta$ CR1 (the truncated form of Cux1) protein in the double knockout mice which in turn repressed p21 and p27 thereby increasing cell proliferation resulting in a rapid progression of the disease (87).

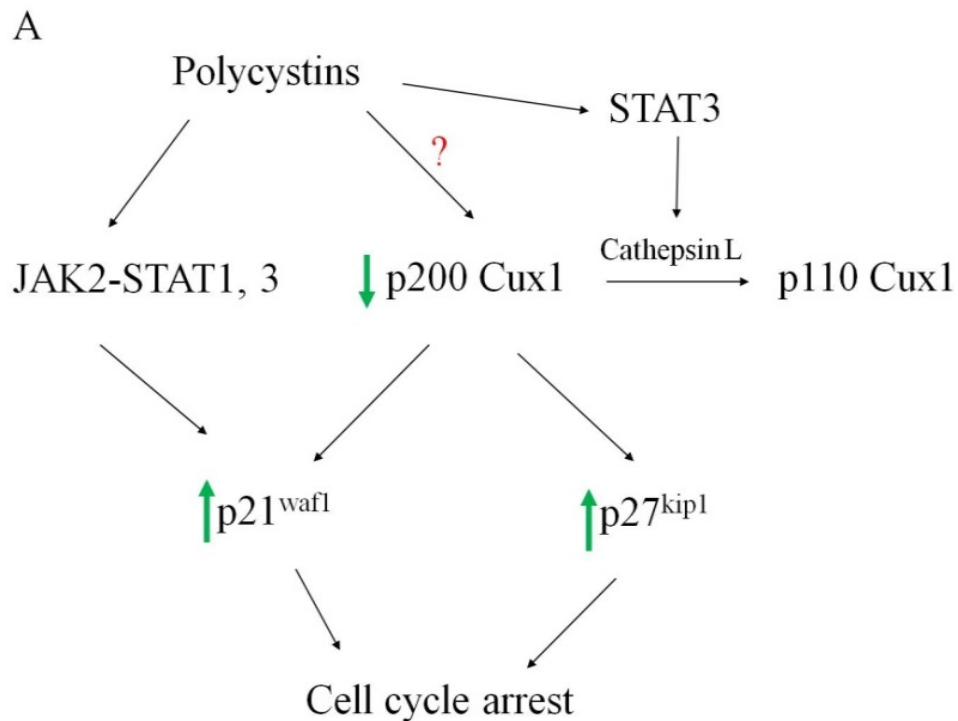
### A regulatory loop between Cux1 and Polycystin-1

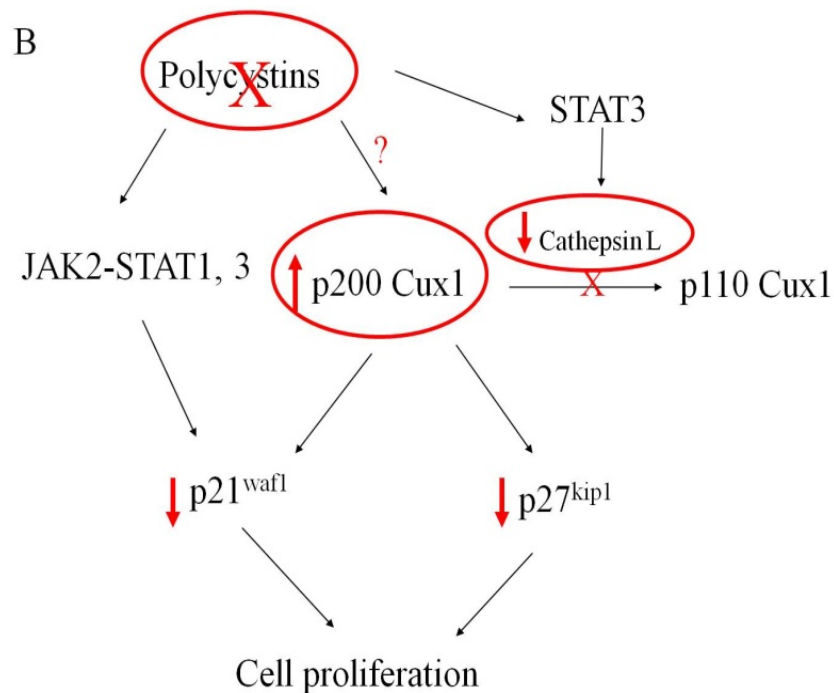
Cux1 regulates cell cycle by inhibiting p21 and p27 CKIs. Cux1 transgenic mice which overexpressed Cux1 had multiorgan hyperplasia including renal hyperplasia. This phenotype was similar to that of the p27 knockout mice which also showed kidney hyperplasia. Both mRNA and protein levels of p27 were downregulated in Cux1 transgenic mice. Moreover, promoter reporter assays showed repression of the p27 promoter by Cux1 (101, 126).

A study by Bhunia *et al* placed polycystins in direct control of the cell cycle. They showed that PC1 binds to JAK2 which then phosphorylates STAT1 and 3 leading to the inhibition of the CKI, p21. Another study by Li *et al* showed that PC2 interacts with an HLH protein Id2 and regulates the cell cycle. Both of these studies suggested that polycystins act to induce cell cycle arrest by activating p21 (48, 49) (model shown in Figure 1-5 G). However, unlike the p27 knockout mice which showed a renal hyperplasia phenotype, the p21 knockout mice did not show any striking phenotype. No renal hyperplasia was observed in these mice (127).

A more recent observation by Talbot *et al* also provided evidence that both membrane-anchored and soluble PC1 C-terminal tails can regulate STATs 1, 3 and 6 (51). Research in the

obesity field shows that cathepsin-L, a proteolytic enzyme which generates shorter isoforms of Cux1, is regulated by STAT3. Moreover, this study also places Cux1 in a ciliary assembly function (128). ADPKD cells show an increase in the accumulation of full length p200 Cux1 which is correlated with a decrease in cathepsin-L levels (87). This evidence shows that Cux1 may be regulated by the polycystin complex (Figure 1-11).





**Figure 1-11 A regulatory loop between Cux1 and Polycystin-1** A: In a normal person who does not have ADPKD, polycystins are normal and through the JAK-STAT pathway p21 is upregulated and cell cycle arrest takes place in differentiated cells. Cathepsin-L processes the full length p200 Cux1, which also leads to a decrease in the CKIs p21 and p27 which leads to cell cycle arrest. B: In a person with ADPKD, polycystin function is abnormal owing to the mutations in the *PKD1* or *PKD2* genes. Also, cathepsin-L levels are decreased in these patients leading to the accumulation of the p200 Cux1. Defects in both these pathways lead to an increase in cell proliferation. A role for STAT3 in regulating cathepsin-L has been shown. PC1 is also shown to regulate STAT3 signaling. Therefore, Cux1 maybe directly regulated by PC1 through STAT signaling. This may lead to an exaggerated cell proliferation response in ADPKD patients.

## Goals of this study

Approximately 600,000 individuals in the U.S and 6-10 million worldwide suffer from ADPKD. The disease leads to End-Stage Renal Disease (ESRD) in most patients (1). A recent study evaluating renal function and healthcare costs in PKD patients found that the total healthcare costs rose precipitously in patients who had an eGFR (estimated glomerular filtration rate) less than 30ml/min. Therefore, strategies that prevent renal dysfunction in these patients can also help to reduce their healthcare costs significantly (129).

At present, there are no specific treatments available to treat ADPKD patients. Rather, the current treatment strategies are focused on addressing symptoms such as hypertension and using dietary modifications to delay the process of ESRD. Several clinical trials are underway which target different aspects of the disease.

Increased cell proliferation is a prominent feature in ADPKD, leading to cystogenesis and modification of the disease progression. Thus, identifying molecular mechanisms underlying the cell proliferative defects in ADPKD may provide therapeutic targets for the treatment of the disease. *Cux1*, being a very important gene in renal development as well as in cell cycle regulation, (both features important in PKD) makes it a very good candidate to study in PKD.

Even though *Cux1* is ectopically expressed in various mouse models and in human ADPKD patients, overexpression of *Cux1* in mice did not result in PKD (101). This suggests that overexpression of *Cux1* by itself is insufficient to develop PKD. In this particular study, we sought to determine if *Cux1* is required to develop PKD in an ADPKD mouse model. The central hypothesis is that the deregulation of *Cux1* is required for the cell proliferation defects seen in ADPKD and that it modifies the ADPKD phenotype. The specific aim was to:



**Determine if *Cux1* is required to develop renal cysts in a mouse model carrying a kidney specific deletion in the *Pkd1* gene.**

1. Characterize mice with a kidney specific deletion in the *Pkd1* gene (chapter 2).
2. Characterize Pkd1 mutant mice carrying a targeted mutation in *Cux1* (chapter 3).

## Chapter Two

Ectopic expression of Cux1 is associated with reduced p27 expression and increased apoptosis during late stage cyst progression upon inactivation of Pkd1 in collecting ducts

Binu M. Paul, Dianne Vassmer, Aaron Taylor, Lynn Magenheimer, Carol G. Carlton, Klaus B. Piontek, Gregory G. Germino and Gregory B. Vanden Heuvel.

### Abstract

Polycystic kidney diseases (PKD) are inherited disorders characterized by fluid-filled cysts primarily in the kidneys. We previously reported differences between the expression of Cux1, p21 and p27 in the cpk and Pkd1 null mouse models of PKD. Embryonic lethality of Pkd1 null mice limits its study to early stages of kidney development. Therefore, we examined mice with a collecting duct specific deletion in the *Pkd1* gene. Cux1 was ectopically expressed in the cyst lining epithelial cells of newborn, P7 and P15 Pkd1<sup>CD</sup> mice. Cux1 expression correlated with cell proliferation in early stages of cystogenesis, however, as the disease progressed, fewer cyst lining cells showed increased cell proliferation. Rather, Cux1 expression in late stage cystogenesis was associated with increased apoptosis. Taken together, our results suggest that increased Cux1 expression associated with apoptosis is a common feature of late stage cyst progression in both the cpk and Pkd1<sup>CD</sup> mouse models of PKD.

## Introduction

The Cux1 transcription factor is involved in the regulation of cell proliferation, differentiation and development (119, 133-136). *Cux1* is a murine homologue of the *Drosophila* gene *Cut*. *Cut* is required for the proper development of malpighian tubules in *Drosophila* which are the insect excretory organs that serve as their primitive kidney (88, 87, 99, 115, 134).

Cux1 is highly expressed in the developing kidney, with highest expression restricted to the nephrogenic zone (88). As development proceeds, the levels of Cux1 decrease with only low levels of Cux1 detected in adult kidneys (101). Cux1 regulates the cell cycle by transcriptionally repressing the cyclin dependent kinase inhibitors (CKI) p21 and p27 (124, 118).

High rates of cell proliferation are one of the striking features of cyst epithelial cells in PKD, a life-threatening genetic disease. PKD can be inherited in two different forms: an autosomal recessive form (ARPKD), or an autosomal dominant form (ADPKD), both characterized by fluid-filled cysts primarily in the kidneys (2). ADPKD results from mutations in either of the two genes, *PKD1* or *PKD2* (2, 50, 137, 138), while mutations in a single locus, *PKHD1*, are responsible for ARPKD (8).

Polycystin1, the protein product of *PKD1* co-localizes with complexes involved in cell-to-cell and cell-to-extracellular matrix interactions. These complexes in turn have a regulatory role in cell proliferation (139). Polycystin1 also interacts with Polycystin2, the protein product of *PKD2*, to induce p21 (48), a transcriptional target of repression by Cux1 (118).

Several murine models have been described for PKD. A well characterized murine model of PKD is the *cpk* mouse model. The disease is transmitted in a recessive fashion and it shows a striking resemblance to human ARPKD in terms of cyst localization and disease progression (3,

140). A targeted mutation in the *Pkd1* gene (*Pkd1* null) has also been described. The *Pkd1* null mice which are homozygous for this mutation present with kidney cysts and die embryonically (60).

Cux1 is upregulated in the kidneys of both the *cpk* and the *Pkd1* null mouse models (124). Cells from human ADPKD kidneys also show increased expression of Cux1 (87). Analysis of *cpk* and the *Pkd1* null mouse models showed a striking difference between the expression of Cux1, p21, p27, as well as, cell proliferation and apoptosis. Kidneys from *Pkd1* null embryos showed increased expression of Cux1. However, in the kidneys of *cpk* mice, Cux1 upregulation was not observed until late stages of cystogenesis. While p21 was not detected in embryonic kidneys from *Pkd1* null mice, Cux1 and p21 were co-expressed in cyst lining cells in *cpk* mice. In contrast to the reduced expression of p27 in kidneys from *Pkd1* null embryos, we saw an increase in p27 expression in the *cpk* kidneys during late stages of cystogenesis. Apoptosis was also increased in the *cpk* kidneys during late stages of cystogenesis (124).

These results suggested a model in which cystogenesis proceeds through different mechanisms in the *Pkd1* null mice and *cpk* mice. However, since the *Pkd1* null mice died embryonically, our analysis of cystogenesis in that mouse model was restricted to the earliest stages of cystogenesis. In order to analyze the role of Cux1 in ADPKD beyond the embryonic stages of cystogenesis, we examined a mouse model with a collecting duct specific deletion of the *Pkd1* gene. Early stages of cystogenesis in this mouse model showed an increase in Cux1 expression that correlated with increased cell proliferation. In more advanced stages of cystogenesis, the increased expression of Cux1 was associated with an increase in apoptosis.

## Experimental Procedures

### Pkd1<sup>cond</sup> mice

The Pkd1<sup>cond</sup> mouse line has been described previously. This mouse line has *loxP* sites flanking exons 2 through 4 of the murine *Pkd1* gene, thereby allowing the inactivation of the gene specifically in the tissue of interest. The Pkd1<sup>cond</sup> allele is fully functional and mice homozygous for this allele are viable and healthy (147).

### Hoxb7/Cre mice

The Hoxb7/Cre transgenic mouse line (STOCK Tg(Hoxb7-cre)13Amc/J) was purchased from Jackson Laboratory (Bar Harbor, ME) and the stock colonies are maintained at the University of Kansas Medical Center. The Cre recombinase in this transgenic mouse is expressed under the control of the mouse Hoxb7 enhancer and promoter. The expression of Hoxb7/ Cre can be detected in the mesonephric duct of the kidney as early as embryonic day 9.5. Thereafter, Cre expression can be seen in the mesonephric duct derivatives of the kidney, which include the collecting duct and the ureteral epithelia (148).

### Breeding strategy

We crossed mice heterozygous for the Pkd1<sup>cond</sup> allele to Hoxb7/Cre<sup>+</sup> mice. Brother-sister matings were set up between F1 progeny having the Pkd1<sup>cond/wt</sup>; Hoxb7/Cre<sup>+</sup> genotype. A total of 59 out of 266 pups were Pkd1<sup>CD</sup> (homozygous for the Pkd1<sup>cond</sup> allele and heterozygous for the Hoxb7/cre transgene). All protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. The University of Kansas Medical Center is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

### Characterization of cystic phenotype

We analyzed the offspring of F1 crosses at postnatal day 0 (P0, newborn), P7, and P15 stages. Kidneys were harvested from newborn (P0), 7-day old (P7), 12-day old (P12) and 15-day old (P15) mice. Kidneys were weighed and the total kidney weight (KW) was measured as a percentage of body weight (BW) for each mouse. Harvested kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Mid-sagittal sections (5µm) of cystic kidneys, stained with Haematoxylin and eosin (H & E), were utilized to stage the cysts, as described previously (87). Cysts with up to 50 cyst-lining epithelial cells were defined as small, medium if there were 51-200 cyst-lining epithelial cells, and advanced (large) stage cysts if there were more than 200 cyst lining epithelial cells. The cystic index (ratio of cystic area in the kidney to the total kidney area) was measured in H & E stained cystic kidney sections using ImageJ (NIH) software.

### Serum chemistry

Blood was collected by exsanguination, following decapitation and immediately centrifuged at 2000 g to collect serum. Blood urea nitrogen (BUN) in these samples was measured using QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA).

### Immunofluorescence (IF)

Immunofluorescence was performed as previously described (124). Kidney sections were incubated with 1M ammonium chloride to quench autofluorescence, washed in PBST, and blocked in 10% normal goat serum (NGS) or 10% normal horse serum (NHS) for 1 hour at room temperature. Rabbit anti-Cux1 (1:50, Santa Cruz), mouse anti-PCNA (1:3000, Sigma), mouse anti-p21 (1:100, AbCam), primary antibody was applied to sections and incubated for 1 hour at

room temperature. Bound anti-Cux1 antibodies were detected using biotinylated goat anti-rabbit (1:400) secondary antibody (Vector) followed by FITC-avidin (5µg/ml, Vector). PCNA and p21 antibodies were detected using horse anti-mouse Texas red or FITC-conjugated secondary antibody (Vector, 1:400). To identify collecting ducts, kidney sections were incubated with biotinylated dolichos biflorus agglutinin (20ug/ml DBA, Vector) for 1 hour at room temperature, followed by incubation with FITC-conjugated avidin. Sections were then washed in PBST, mounted with Vectashield medium with DAPI (Vector) and images were captured with an Optronics Magnafire digital camera.

#### Western blot analysis

Whole tissue lysates were prepared from frozen kidneys. Protein (40ug) was loaded onto 4-15% or 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% milk in PBST. Membranes were probed with anti-p27 antibody (1:100, AbCam), or anti-β-tubulin antibody (1:1000, Sigma), followed by PBST washes and HRP-peroxidase secondary antibody (1:10,000) application.

#### TUNEL assay

Sections were processed for Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) with the TUNEL Apoptosis Detection Kit (Upstate) according to manufacturer's instructions. Sections were counterstained with DAPI, coverslipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera.

## Statistics

Two-tailed t-tests were performed in all statistical studies.

## Results

Our previous studies with *cpk* and *Pkd1* null mice suggested different mechanisms of PKD progression in these mouse models. These studies showed that increased expression of *Cux1* was primarily associated with cell proliferation in the *Pkd1* null mice. In contrast, increased expression of *Cux1* in the *cpk* mice during late stages of cyst progression was associated with apoptosis (124).

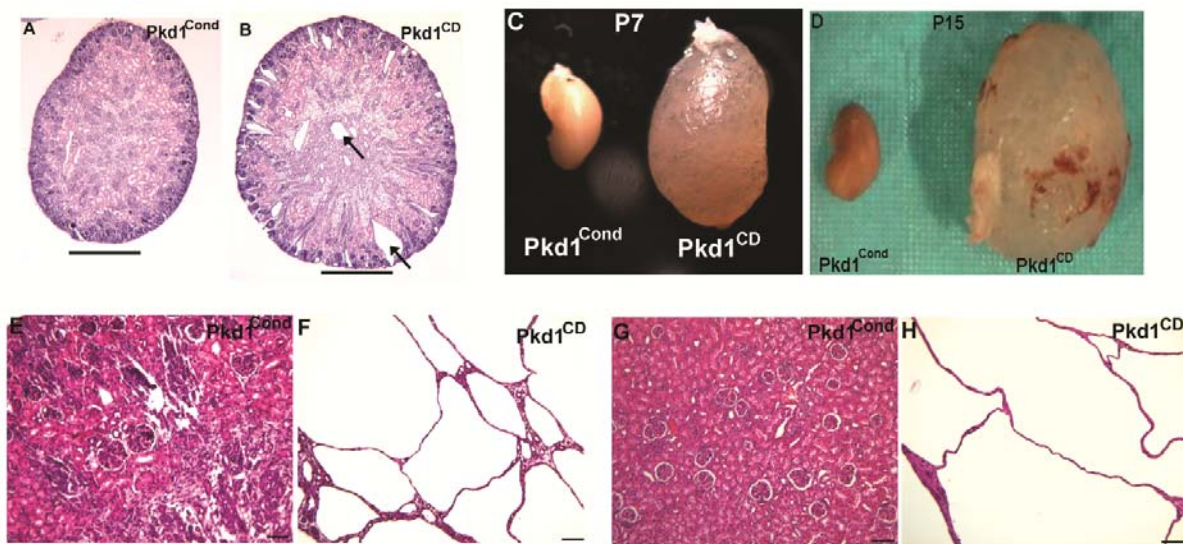
Embryonic lethality of *Pkd1* null mice limited our studies to the early stages of cystogenesis. Therefore, in the present study, we have examined an ADPKD mouse model with a conditional deletion of the *Pkd1* gene in the kidneys. We crossed the *Pkd1*<sup>cond</sup> mice with *Hoxb7*/Cre mice to generate a kidney specific deletion of the *Pkd1* gene. *Hoxb7*/Cre is active in the mesonephric duct of the kidney as early as embryonic day 9.5 and its expression continues in the mesonephric duct derivatives of the kidney, which include collecting ducts and ureteral epithelia (148). Mice in which the *Pkd1* gene was disrupted using *Hoxb7*/cre were designated *Pkd1*<sup>CD</sup> (*Pkd1*<sup>collecting duct</sup>) mice.

## Morphological evaluation of the *Pkd1*<sup>CD</sup> mice

We analyzed *Pkd1*<sup>CD</sup> mice at various ages, beginning at postnatal day 0 (P0). Mice with *Hoxb7*/Cre<sup>+</sup>/*Pkd1*<sup>cond/wt</sup> or *Hoxb7*/Cre<sup>+</sup>/*Pkd1*<sup>wt/wt</sup> genotypes were used as controls. Morphological analysis of kidney sections from newborn *Pkd1*<sup>CD</sup> mice revealed microscopic cysts. These microscopic dilations were derived from both cortical and medullary collecting ducts (Figure 2-

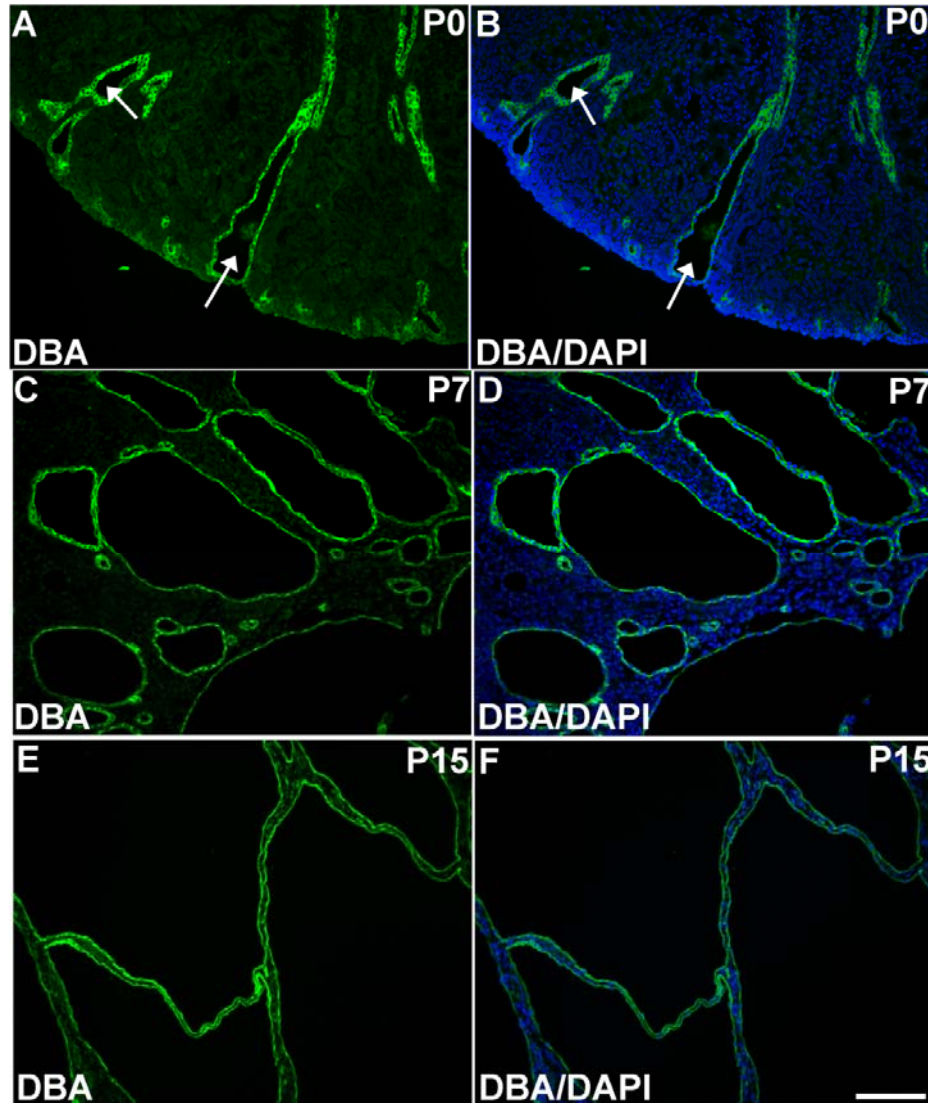


1A). In mice, nephrogenesis continues until about postnatal day 7 (P7) (28). Therefore, we analyzed Pkd1<sup>CD</sup> mice at this time point. By P7, Pkd1<sup>CD</sup> mice had a slight bulging of their flanks, which was visible only upon careful examination. The kidneys of these mice were larger and cystic compared to their age-matched control littermates (Figure 2-1B). The kidneys also had more and larger cysts, with less normal parenchyma, compared to cystic kidneys isolated from newborn Pkd1<sup>CD</sup> mice (Figure 2-1E-F). By P15, Pkd1<sup>CD</sup> mice presented with huge bilateral masses on their flanks. The kidneys of these mice were larger and grossly cystic in comparison to age-matched control littermates, or when compared to Pkd1<sup>CD</sup> mice at P0 and P7 (Figure 2-1D). The kidneys were crowded by cystic tissue and very little normal renal parenchyma was preserved (Figure 2-1G-H).



**Figure 2-1 Morphological evaluation of the Pkd1<sup>CD</sup> mice.** A and B: Bright field images of newborn kidneys from a Pkd1<sup>CD</sup> (B) mouse and an age-matched control littermate (A) Microscopic cyst dilations (arrows) can be seen in the Pkd1<sup>CD</sup> kidney. Scale bar= 500µm. C and D: Gross appearance of littermate kidneys (control and Pkd1<sup>CD</sup>) at P7 (C) and P15 (D) respectively. The Pkd1<sup>CD</sup> kidneys are large and severely cystic compared to the controls. Pkd1<sup>CD</sup> kidney at P15 is also larger compared to the Pkd1<sup>CD</sup> kidney at P7. E-H: H & E stained kidney sections from control and Pkd1<sup>CD</sup> mice at P7 (E-F) and P15 (G-H) respectively. By P15, the Pkd1<sup>CD</sup> kidneys lost most of the normal renal parenchyma and the kidneys were crowded with cystic tissue. Scale bar= 100µm.

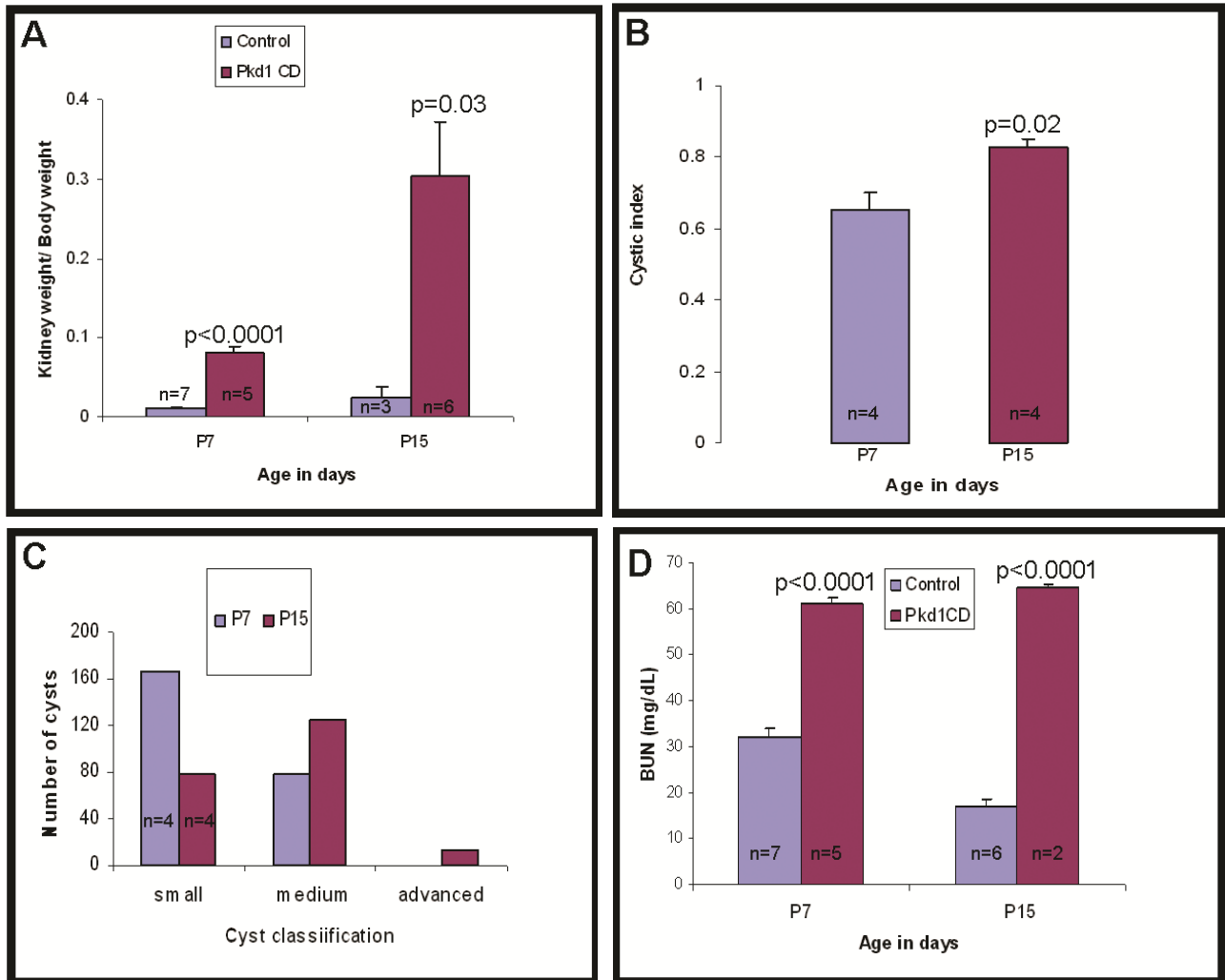
Since HoxB7/Cre specifically deletes the *Pkd1* gene in the ureteric bud derivatives of the kidney, we expected all the cysts in the *Pkd1*<sup>CD</sup> mice to have originated from the collecting ducts. Labeling of kidney sections from newborn, P7 and P15 *Pkd1*<sup>CD</sup> mice with Dolichus Biflorus Agglutinin (DBA), confirmed the collecting duct origin of the cysts (Figure 2-2 A-F).



**Figure 2-2: All the cysts in the *Pkd1*<sup>CD</sup> kidneys originated from collecting ducts.** Kidney sections from *Pkd1*<sup>CD</sup> mice were labeled with DBA (green, A, C and E) and merged with DAPI staining (blue, B, D and F). Arrows show some collecting duct derived cysts labeled with DBA. Arrowheads show normal collecting ducts labeled with DBA. A, B: Kidney sections from a newborn *Pkd1*<sup>CD</sup> mouse. C, D: Cystic kidney from a *Pkd1*<sup>CD</sup> mouse at P7. E, F: Cystic kidneys from a *Pkd1*<sup>CD</sup> mouse at P15. Scale bar= 100μm.

### Analysis of PKD severity in the Pkd1<sup>CD</sup> mice

To evaluate disease progression in Pkd1<sup>CD</sup> mice, we determined the ratio of kidney weight to body weight (KW/BW) at P7 and at P15 (Figure 2-3A). Kidneys harvested from Pkd1<sup>CD</sup> mice were significantly larger compared with kidneys from control mice, both at P7 and P15. To further analyze the Pkd1<sup>CD</sup> cystic phenotype, isolated cystic kidneys were examined morphologically. Histological analysis showed that the cystic index (the percentage of cystic area) increased between P7 and P15 (Figure 2-3B). The developmental stage of the renal cysts in Pkd1<sup>CD</sup> mice was determined by counting the number of cells lining the cysts (see experimental procedures). The results showed that cystic kidneys in P7 mice were mainly composed of small and medium cysts, while advanced stage cysts were also seen in the cystic kidneys of P15 mice (Figure 2- 3C). Cystic kidney disease is directly correlated with reduced renal function and high BUN levels. Accordingly, Pkd1<sup>CD</sup> mice at P7 and P15 showed higher BUN values compared to controls, indicative of decreased renal function (Figure 2- 3D).

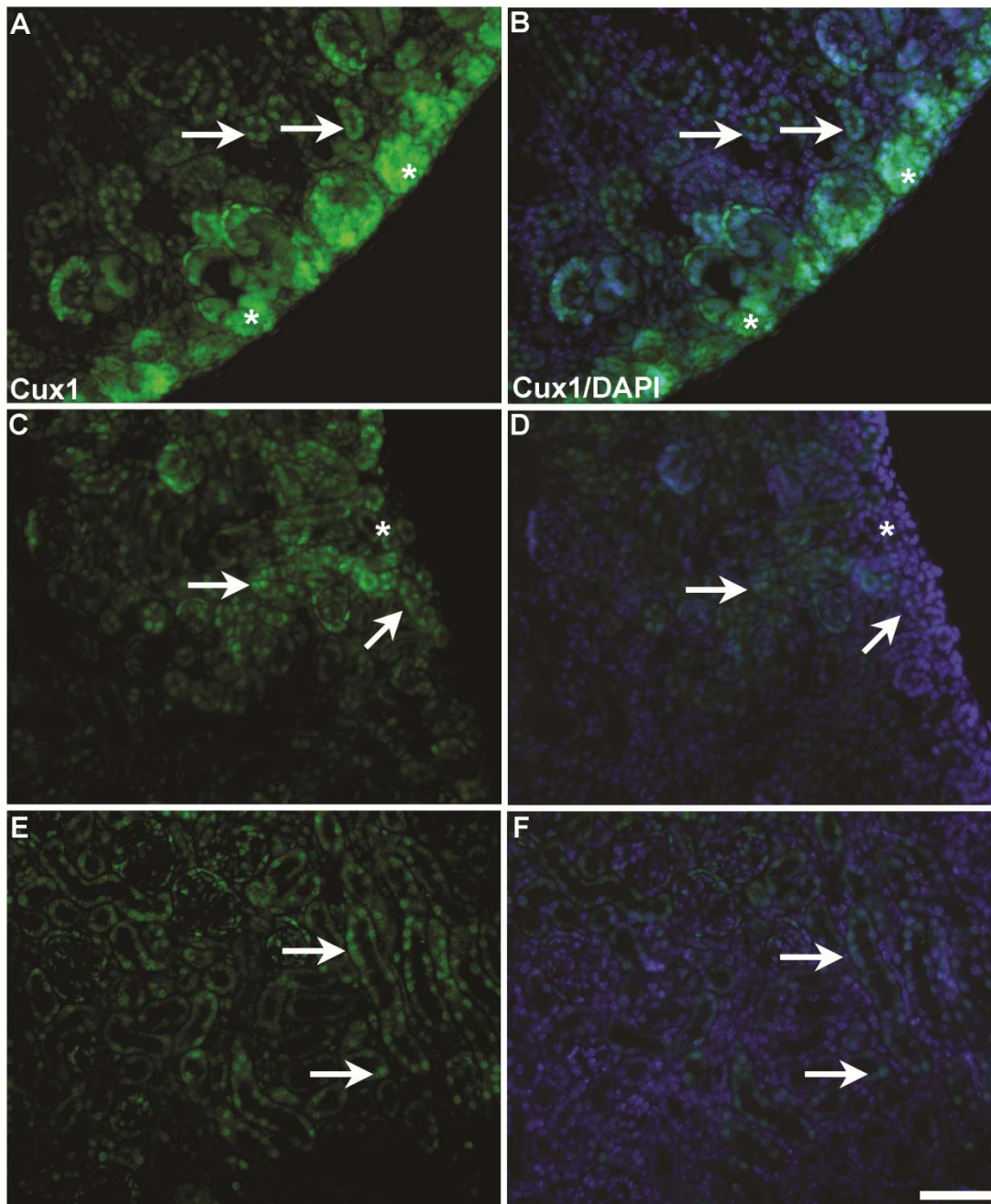


**Figure 2-3: Analysis of PKD severity in the Pkd1<sup>CD</sup> mice.** A: KW/BW of P7 and P15 Pkd1<sup>CD</sup> mice compared to their respective age-matched controls. KW/BW was significantly increased in P7 (p value <0.0001) and P15 (p value 0.03) Pkd1<sup>CD</sup> mice compared to their age-matched controls. B: Cystic index of kidneys from Pkd1<sup>CD</sup> mice at P7 and P15 are shown. Cystic index was significantly higher in P15 kidneys compared to P7 kidneys from Pkd1<sup>CD</sup> mice (p value 0.02). C: Cysts classified according to their developmental stages are shown. Small cysts had up to 50 cyst-lining epithelial cells. Cysts which had 51-200 cyst-lining epithelial cells were defined as medium cysts. Advanced stage or large cysts were lined with more than 200 cyst-lining epithelial cells. By P7, Pkd1<sup>CD</sup> kidneys were comprised mainly of small cysts. Medium cysts were also seen in these cystic kidneys. However, no advanced cysts were seen in these kidneys. The Pkd1<sup>CD</sup> kidneys at P15 showed more medium cysts and also a small percentage of advanced stage cysts. D: BUN values from the Pkd1<sup>CD</sup> mice were compared with their age-matched controls. Increased BUN values were observed in Pkd1<sup>CD</sup> mice at both P7 (p value <0.0001) and P15 (p value <0.0001) compared to their respective age matched controls. Between P7 and P15, BUN values did not show a great difference.

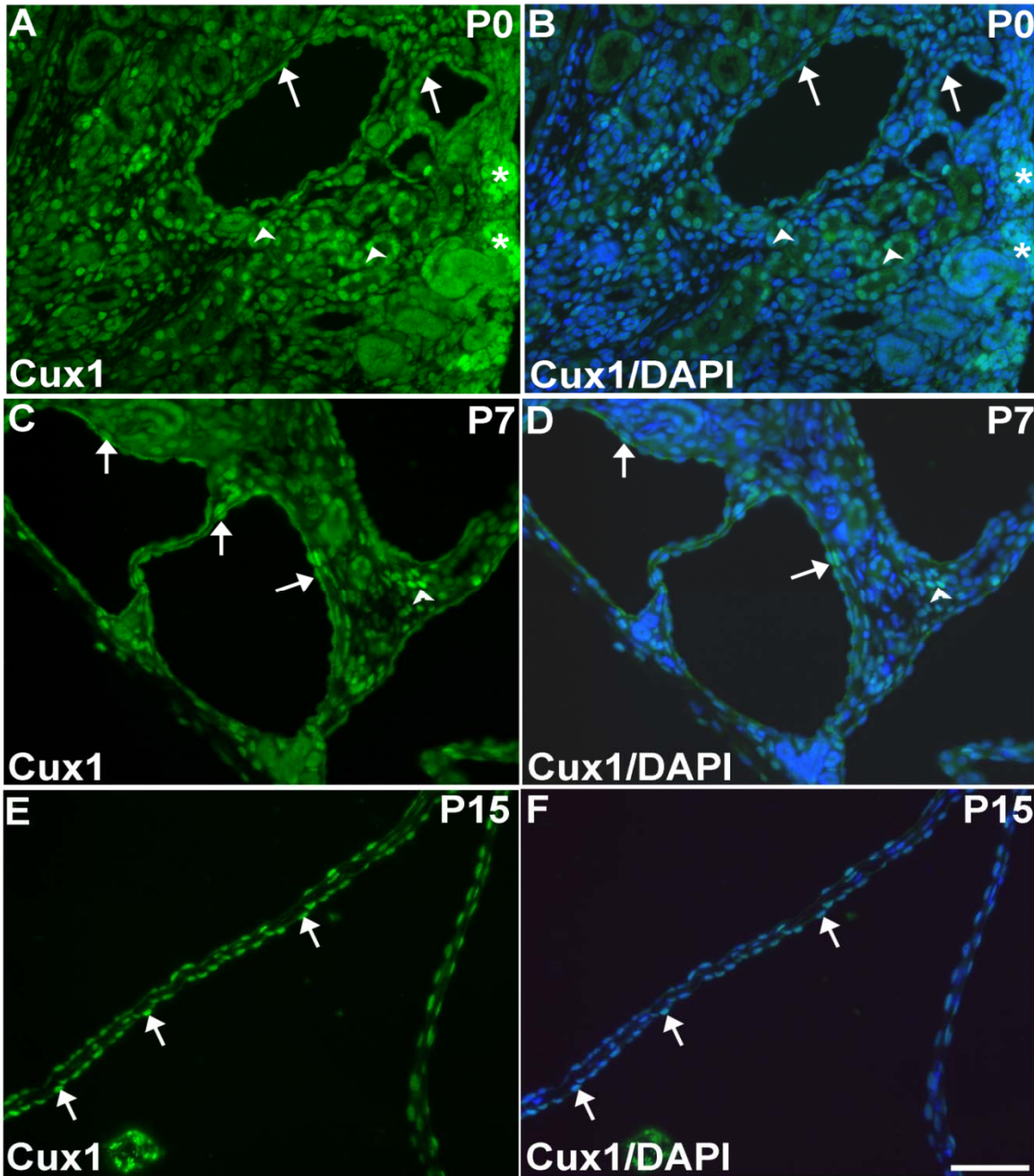
Cux1 is ectopically expressed in the Pkd1<sup>CD</sup> mice.

Cux1 is highly expressed during normal kidney development with the highest level of expression seen in the nephrogenic zone of the kidney (88, 124). Since Cux1 is a cell cycle regulatory gene and increased cell proliferation is a hallmark characteristic of PKD, we analyzed the expression pattern of Cux1 at various stages of cystogenesis in the Pkd1<sup>CD</sup> mice. As expected, high levels of Cux1 were seen in the nephrogenic zone of newborn control kidneys (Figure 2-4 A-B), as well as in the Pkd1<sup>CD</sup> kidneys (Figure 2-5 A-B). Cux1 was also ectopically expressed in the cyst lining epithelium of kidneys from Pkd1<sup>CD</sup> mice (Figure 2-5A-B). The continuation of the proliferative phase of kidney development at P7 correlated with continued expression of Cux1 in the kidneys of control mice (Figure 2-4 C-D). Cystic kidneys from P7 Pkd1<sup>CD</sup> mice showed increased expression of Cux1, (Figure 2-5 C-D) compared to the controls. By P15, control kidneys showed little Cux1 expression (Figure 2-4). In contrast, cystic kidneys from Pkd1<sup>CD</sup> mice continued to show high and ectopic expression of Cux1 (Figure 2-5 E-F).





**Figure 2-4: Cux1 expression in the control kidneys.** Kidney sections from control mice were labeled with Cux1 (green, A, C and E) and merged with DAPI staining (blue, B, D and F). A, B: Kidney section from a newborn mouse. Cux1 labeling was seen in the nephrogenic zone (\*) and tubules (arrows). C, D: Kidney section from a P7 control mouse. Cux1 staining in the nephrogenic zone (\*) is decreased as compared to the newborn mouse. Cux1 was also seen in some tubules (arrows). E, F: Kidney section from a P15 control mouse. Cux1 levels are much reduced by P15 compared to the newborn and P7 control kidneys. Arrows point toward Cux1 positive nuclei. Scale bar= 50µm.

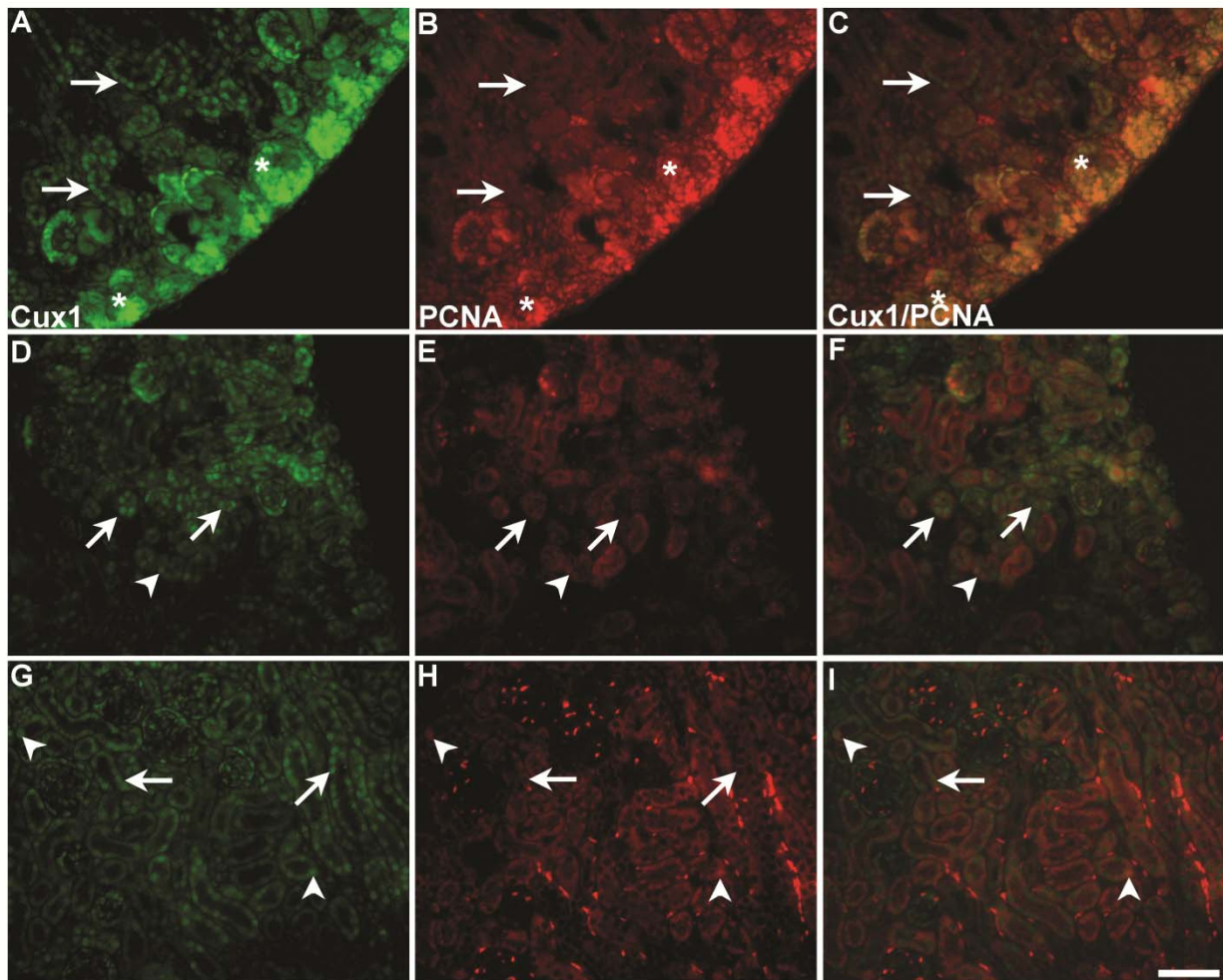


**Figure 2-5: Ectopic expression of Cux1 in the  $Pkd1^{CD}$  kidneys.** Kidney sections from  $Pkd1^{CD}$  mice were labeled with Cux1 (green, A, C and E) and merged with DAPI staining (blue, B, D and F). A, B: Kidney section from a newborn  $Pkd1^{CD}$  mouse. Cux1 labeling was seen in the nephrogenic zone (\*) and cysts (arrows). C, D: Cystic kidney section from a P7  $Pkd1^{CD}$  mouse. Arrows point toward Cux1 positive nuclei in the cysts. Arrowheads show normal tubules positive for Cux1. E, F: Cystic kidney section from a P15  $Pkd1^{CD}$  mouse. Arrows point toward Cux1 positive nuclei in the cysts. All the cyst-lining epithelial cells were Cux1 positive. Scale bar= 50 $\mu$ m.

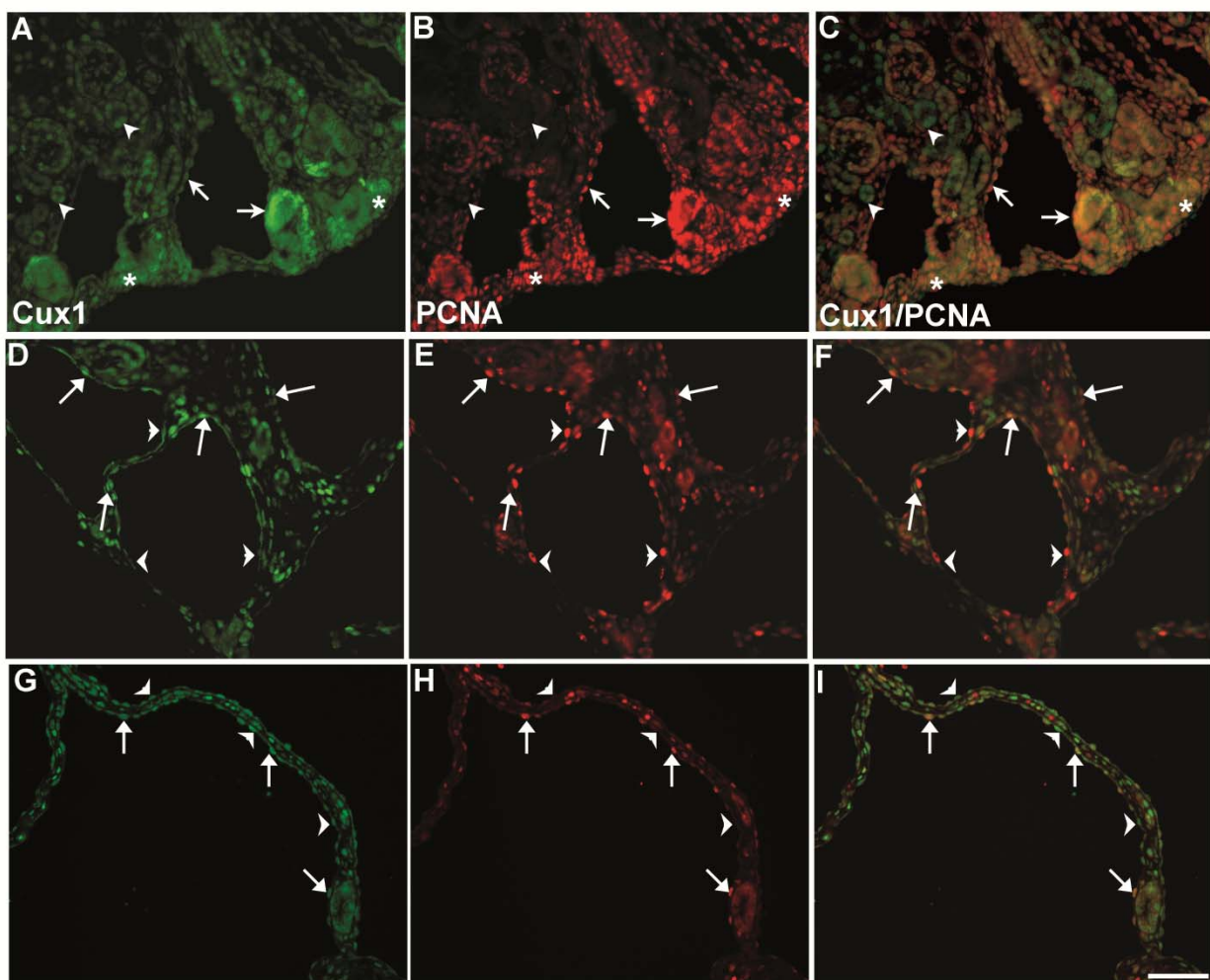
Early and late stage of cystogenesis in the Pkd1<sup>CD</sup> mice is associated with increased cell proliferation and increased Cux1 expression.

Increased cell proliferation is one of the characteristic features of PKD. We have previously shown that increased expression of Cux1 is associated with increased cell proliferation in human ADPKD cystic epithelia and in several mouse models of PKD (87, 124). We analyzed cell proliferation and its association with Cux1 in the Pkd1<sup>CD</sup> mice by labeling kidney sections for Cux1 and the cell proliferation marker PCNA. PCNA staining co-localized with Cux1 in the nephrogenic zone and in the cyst lining cells of newborn and P7 Pkd1<sup>CD</sup> mice (Figure 2-7). By P15, the nephrogenic zone is essentially gone, however, the cyst lining cells expressed PCNA and Cux1 (Figure 2-7 G-I). Kidney sections from control newborn mice showed high levels of cell proliferation, which were associated with Cux1 expression. In contrast, kidney sections from P7 and P15 control mice showed little PCNA or Cux1 expression (Figure 2-6 A-I).





**Figure 2-6: Decreased Cux1 expression and cell proliferation in the control kidneys.** Kidney sections from control mice were co-labeled with Cux1 (green, A, D and G), and PCNA (red, B, E and H). A merge between Cux1 and PCNA is also shown (C, F and I). A, B, C: Newborn control kidney section. Cux1 and PCNA co-localized in the nephrogenic zone (\*). Arrows point toward tubules which show no co-localization between Cux1 and PCNA. D, E, F: Kidney section from a P7 control mouse. Very few tubules stained positive for PCNA. Cux1 and PCNA co-localized in some tubules (arrowheads). Arrows point toward tubules in which Cux1 and PCNA did not co-localize. G, H, I: Kidney section from a P15 control mouse. Very few tubules stained positive for PCNA. Cux1 and PCNA co-localized in some tubules (arrowheads). Arrows point toward tubules in which Cux1 and PCNA did not co-localize. Scale bar= 50 $\mu$ m.

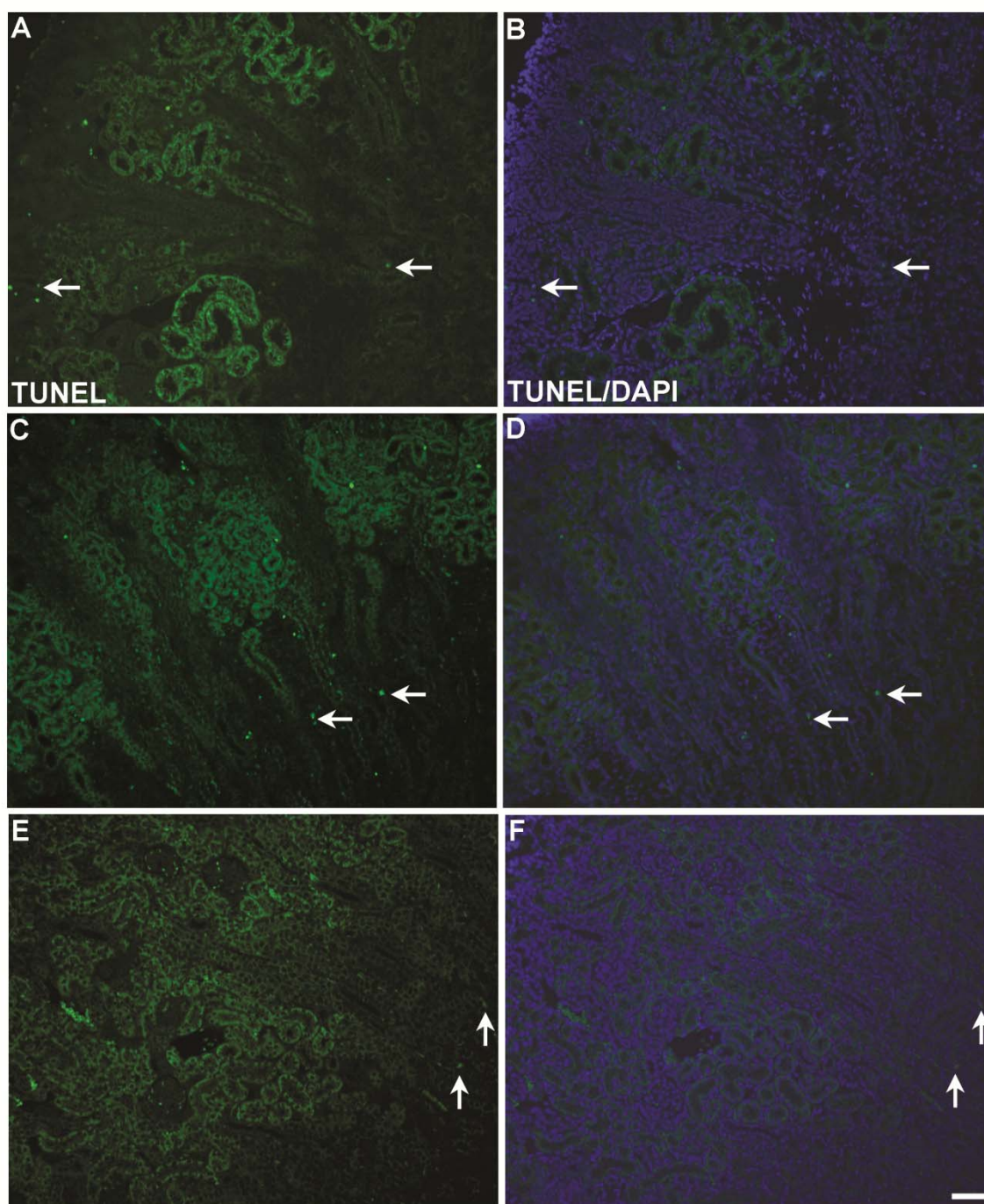


**Figure 2-7: Early and late stage cystogenesis in Pkd1<sup>CD</sup> mice is associated with increased cell proliferation.** Kidney sections from Pkd1<sup>CD</sup> mice were co-labeled with Cux1 (green, A, D and G), and PCNA (red, B, E and H). A merge between Cux1 and PCNA is also shown (C, F and I). A, B, C: Newborn Pkd1<sup>CD</sup> kidney section. Cux1 and PCNA co-localized in the nephrogenic zone (\*). Arrows point toward nuclei in the cysts which show co-localization of Cux1 and PCNA. Arrowheads show normal tubules where Cux1 and PCNA did not co-localize. D, E, F: Kidney section from a P7 Pkd1<sup>CD</sup> mouse. Arrows point toward nuclei in the cysts which show co-localization of Cux1 and PCNA. Arrowheads show cystic nuclei in Cux1 and PCNA did not co-localize. G, H, I: Kidney section from a P15 Pkd1<sup>CD</sup> mouse. All of the cyst-lining epithelial cells were positive for Cux1. However, only some of them showed co-localization with PCNA (arrowheads). Arrows point toward the co-localization of Cux1 and PCNA in cyst-lining epithelial cells. Scale bar= 50µm.

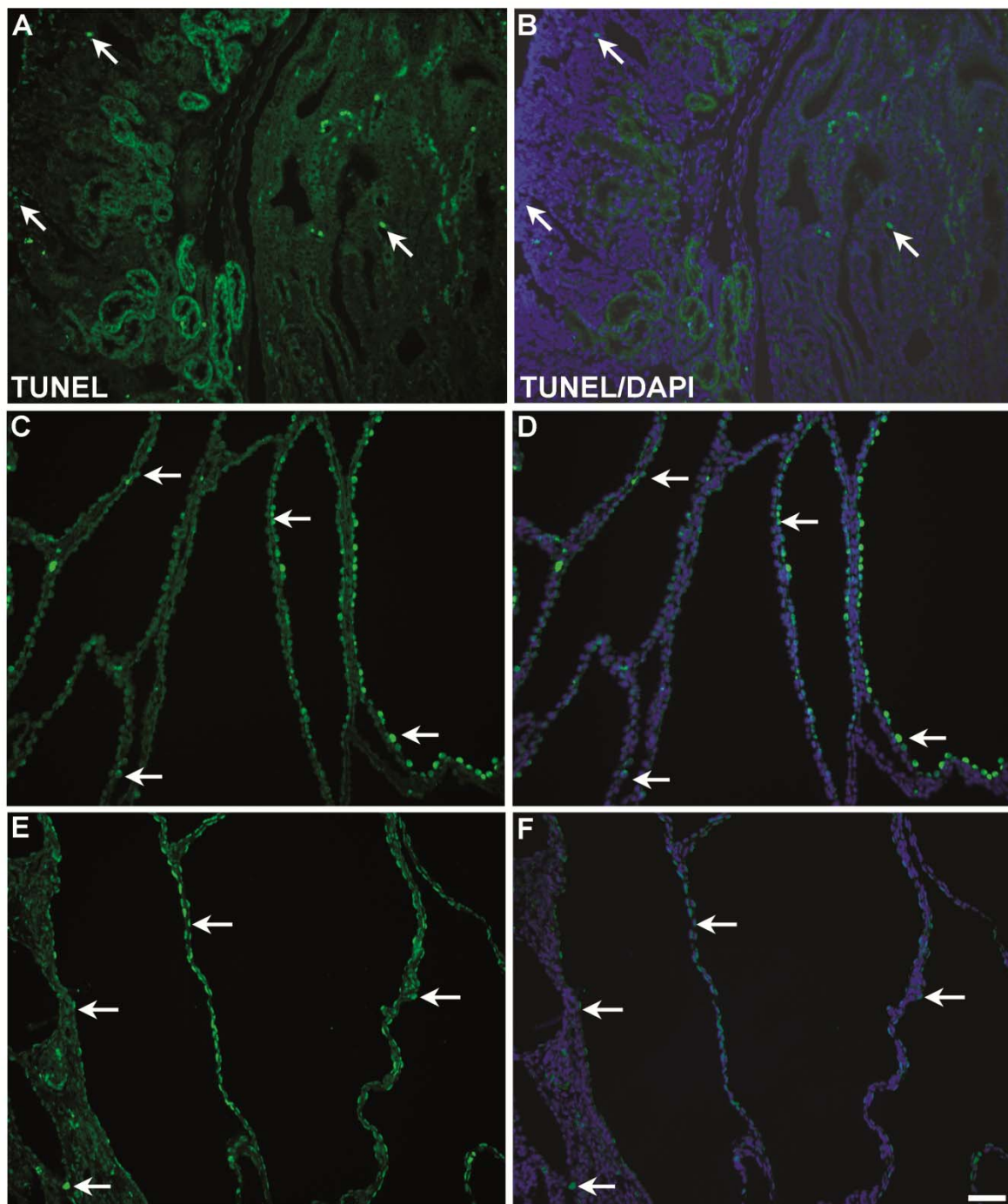
Late stage of cystogenesis in the Pkd1<sup>CD</sup> mice is associated with increased apoptosis and increased Cux1 expression.

Apoptosis is another pathological feature seen in PKD (24). We used the TUNEL assay to analyze apoptosis in kidney sections from Pkd1<sup>CD</sup> mice. Kidney sections from newborn Pkd1<sup>CD</sup> mice were mostly TUNEL negative (Figure 2-9A), while kidney sections from P7 and P15 Pkd1<sup>CD</sup> mice showed increased apoptosis (Figure 2-9 B-C). In contrast, age-matched littermate controls showed little or no apoptosis (Figure 2-8).





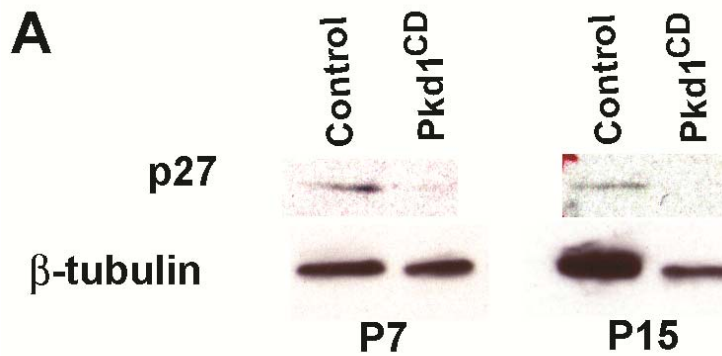
**Figure 2-8: Decreased apoptosis in the control kidneys.** Kidney sections from control mice were stained with TUNEL kit (green, A, C and E) to label apoptotic nuclei and were merged with DAPI (blue, B, D and F) staining. Very few apoptotic nuclei (arrows) were seen in control mice. A, B: Newborn control kidney section. C, D: Kidney section from a P7 control mouse. E, F: Kidney section from a P15 control mouse. Scale bar= 50 $\mu$ m.



**Figure 2-9: Late stage cystogenesis in the Pkd1<sup>CD</sup> mice is associated with increased apoptosis.** The terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) method was used to identify apoptotic cells in newborn (A), P7 (B) and P15 (C) kidneys from Pkd1<sup>CD</sup> mice. A: Newborn Pkd1<sup>CD</sup> kidney section. Very few apoptotic nuclei (arrows) were seen in the newborn kidneys. B: Kidney section from a P7 Pkd1<sup>CD</sup> mouse showing cyst-lining epithelial cells positive for TUNEL labeling (arrows). C: Kidney section from a P15 Pkd1<sup>CD</sup> mouse showing cyst-lining epithelial cells that are TUNEL positive (arrows). Scale bar= 50µm.

Increased expression of Cux1 correlates with the down regulation of p27 in the Pkd1<sup>CD</sup> mice.

Since p27 and p21 are targets of transcriptional repression by Cux1, we analyzed the levels of these proteins in total kidney lysates from the Pkd1<sup>CD</sup> mice. Compared to controls, p27 levels were reduced in the kidneys from both P7 and P15 Pkd1<sup>CD</sup> mice (Fig 2-10 A). p21 is normally downregulated very early during kidney development. Accordingly, we were unable to detect p21 expression in the Pkd1<sup>CD</sup> mice (data not shown).



**Figure 2-10 Downregulation of p27 in the Pkd1<sup>CD</sup> mice.** A: Total kidney lysates were prepared from P7 and P15 Pkd1<sup>CD</sup> mice and their respective age matched controls. A western blot analysis was done on these lysates using antibodies raised against p27 and β-tubulin (loading control). A loss of p27 protein expression was seen in the Pkd1<sup>CD</sup> mice.

## Discussion

Polycystic kidney disease is a systemic disorder characterized by fluid-filled renal cysts together with several extra-renal features. Autosomal dominant polycystic kidney disease (ADPKD) results from mutations in one of two genes: *PKD1*, which encodes the polycystin-1 protein, and *PKD2*, which encodes the polycystin-2 protein. Increasing evidence suggests that PKD is a developmental disorder (60, 141). Aberrant cell proliferation is a pathological feature of PKD and micropolyps or foci of proliferating cells can be found populating the kidneys of

human PKD patients and experimental animal models of PKD (87, 142-144). The role of polycystins in regulating the cell cycle has been described in which polycystin 1, in cooperation with polycystin 2, functions to regulate the cyclin kinase inhibitor p21 by signaling through the JAK-STAT pathway (48).

*Cux1* is a homeobox gene that regulates the cell cycle by transcriptionally repressing the cyclin kinase inhibitors p21 (118) and p27 (104, 124). In the developing kidney, *Cux1* is highly expressed in the nephrogenic zone, an area of high cell proliferation, where it functions to repress p27, thereby keeping cells in the cell cycle. As nephrons mature, the levels of *Cux1* decrease, and cells move out of the cell cycle and terminally differentiate. Our previous studies showed the ectopic expression of *Cux1* in the *Pkd1* null and *cpk* mouse models of polycystic kidney disease (124), and in cells obtained from the renal cysts of ADPKD patients (87). Comparative studies of the expression of *Cux1* and its correlation with cyst progression were done in the *Pkd1* null and *cpk* mouse models. Kidneys from the *Pkd1* null mice showed increased expression of *Cux1*, which correlated with increased cell proliferation. In contrast, increased expression of *Cux1* during late stages of cyst progression in the *cpk* mice was associated with apoptosis. These studies suggested a difference in the mechanism of cyst progression between these animal models. However, the embryonic lethality of *Pkd1* null mice limited our studies to the embryonic stages of cystogenesis. Analysis of the *Pkd1*<sup>CD</sup> mice has allowed us to examine cyst progression in a postnatal ADPKD mouse model.

Microscopic cysts derived from both cortical and medullary collecting ducts were observed in the kidneys from newborn *Pkd1*<sup>CD</sup> mice. Even though the deletion of the *Pkd1* gene was restricted to the collecting ducts, *Pkd1*<sup>CD</sup> mice developed severe PKD as early as P7 where the entire kidney was crowded by cystic tissue, and ectopic expression of *Cux1* was seen in the

kidneys of newborn and P7 Pkd1<sup>CD</sup> mice, where it was associated with cell proliferation. Cux1 was widely expressed in the cyst lining cells from P15 Pkd1<sup>CD</sup> kidneys, however, it did not colocalize with PCNA in many of the cyst lining cells. This apparent uncoupling of Cux1 expression and cell proliferation is similar to what was previously seen in the cpk mice. However, the kidneys of cpk mice did not show increased expression of Cux1 until late stages of cystogenesis.

Consistent with the increased expression of Cux1, we saw downregulation of p27 in the Pkd1<sup>CD</sup> kidneys, similar to what we previously reported for the Pkd1 null mouse. This also contrasts the previously reported upregulation of p21 and p27 in cystic kidneys from cpk mice.

Apoptosis is associated with several mouse models of cystic disease (145). While cystic kidneys from newborn Pkd1<sup>CD</sup> mice did not show higher levels of apoptosis than controls, there was an increase in apoptosis as the disease progressed, similar to our observations in the cpk mouse model. In the cpk mice, there was co-localization of p21 and Cux1 that was associated with increased apoptosis, suggesting contradictory signals to proliferate or arrest (124). However, we did not see ectopic expression of p21 in the Pkd1<sup>CD</sup> mice. While conditional deletion of Pkd1 using Ksp Cre did not show significant changes in apoptosis (146), we did see some TUNEL labeling at very advanced stages of cyst growth, although it seemed to be restricted to only a small subset of large cysts. Thus, although there appears to be apoptosis in the very large cysts, it is probably not involved in the cystogenic process, but in the loss of already damaged cells.

Taken together, our studies support our previous conclusion that differences exist in the mechanism of cyst progression in the Pkd1<sup>CD</sup> and cpk mouse models of PKD. The association



between the expression of Cux1 and cell proliferation is well established. However, the association between Cux1 and apoptosis remains to be elucidated.

## Chapter Three

### Cux1 is required for polycystic kidney disease progression in an ADPKD mouse model

Binu M. Paul, Lynn Magenheimer, Carol G. Carlton and Gregory B. Vanden Heuvel

#### Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the most common monogenic hereditary disorders in humans characterized by fluid-filled cysts, primarily in the kidneys. We previously reported an ADPKD mouse model with a collecting duct specific deletion in the *Pkd1* gene ( $Pkd1^{CD}$ ). These mice developed severe PKD by postnatal day 7 (P7) and died between P15 and P21. *Cux1*, a cell cycle regulatory gene, important during kidney development, was ectopically expressed in the cyst-lining cells of the  $Pkd1^{CD}$  mice. The expression pattern of *Cux1* also correlated with the downregulation of its transcriptional repression targets, p21 and p27. Our results using the *Cux1* transgenic mouse showed that the overexpression of *Cux1* by itself is insufficient to develop PKD. Here we report a double mutant mouse model which has a conditional deletion of the *Pkd1* gene in the renal collecting ducts together with a targeted mutation in the *Cux1* gene ( $Pkd1^{CD}/Cux1\Delta HD$ ). Compared to the  $Pkd1^{CD}$  mice, the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice presented with a reduction in the severity of PKD and also had a longer life span. The delay in the development of PKD in these double mutant mice can be explained by the reduced gene dosage of *Cux1* with a concomitant expression of its transcriptional repression target, p27.

## Introduction

Polycystic Kidney Disease (PKD) is grouped under a set of nephropathies which often progresses to end-stage renal disease (3). It is characterized by the accumulation of fluid-filled renal cysts as well as cysts in some other epithelial organs. Cysts can be defined as fluid-filled structures that are lined with polarized epithelium (144). Renal cysts arise from the epithelia of nephrons and the renal collecting system. The single layer of cells which line the cysts show higher rates of cellular proliferation (2).

PKD is inherited in two different forms, as an autosomal recessive form (ARPKD), or as an autosomal dominant form (ADPKD) (2). ADPKD is a systemic disorder that can have an adult as well as *in utero* onset (12). ADPKD is genetically heterozygous. Mutations in either of two genes, *PKD1* or *PKD2*, can cause ADPKD. Protein products of the *PKD1* (polycystin-1) and *PKD2* (polycystin-2) genes are collectively known as polycystins (2).

Polycystins regulate the cell cycle by activating the cyclin dependent kinase inhibitor (CKI) p21, signaling through the JAK-STAT and Id2 pathways (48-49). The CKI, p21, is also a transcriptional repression target of *Cux1* (118), a cell cycle regulatory gene important during kidney development.

*Cux1* is the murine homologue of the *Cut* gene in *Drosophila melanogaster*. Other mammalian homologues of *Drosophila* *Cut* include CCAAT displacement protein/ CDP/ CUX1 (human), Clox (dog), and CDP2 (rat) (97, 99). In *Drosophila*, *Cut* is required for the proper development of the insect excretory organ, Malpighian tubules. Malpighian tubules serve as the primitive kidneys in these organisms. In murine kidneys, *Cux1* is highly expressed during development and the highest expression is found in the nephrogenic zone which has actively

proliferating cells (88). Cux1 is down-regulated as development ceases and only low levels of Cux1 are found in adult kidneys (101).

There are five evolutionarily conserved domains in the Cut proteins. These include a region that forms a coiled-coil structure, three Cut repeats, and the Cut homeodomain (HD) (98). The Cut repeats, called CR1, CR2 and CR3, each contain approximately 70 amino acids, and along with the homeodomain, are each capable of binding DNA (98, 149). Cux1 functions as a cell cycle dependent transcriptional repressor by negatively regulating the expression of the above-mentioned CKI, p21, and also of another CKI, p27 (101).

Consistent with the transcriptional repression activity of Cux1 on p27, a mouse model which lacks p27, has a similar phenotype as the Cux1 transgenic mouse. Both the Cux1 transgenic and the p27 knockout mice present with multiorgan hyperplasia including renal hyperplasia (101, 126, 150-151). In contrast, p21 knockout mice do not show any kidney hyperplasia (127).

Sinclair et al reported the  $cux/CDP^{\Delta HD/\Delta HD}$  mutant mouse model which has a deletion in the C-terminal domain of the Cux1 protein (122). This deletion generates a mutant Cux1 protein which has a loss of its homeodomain, repression domain and nuclear localization signal (NLS). The mutant Cux1 protein is excluded from the nucleus and is sequestered in the cytoplasm from the loss of its NLS. Hence homozygous  $cux/CDP^{\Delta HD/\Delta HD}$  mutants have a functional loss of Cux1 (122-123).

The role of Cux1 in PKD has been established previously. Cux1 is ectopically expressed in the cyst epithelium of different mouse models of PKD, but is expressed in only very low levels in phenotypically normal littermates (88, 124). In addition, Cux1 is ectopically expressed

in human ADPKD cells (87). *Cux1* transgenic mice which overexpress Cux1 under the control of a CMV promoter, develop multiorgan hyperplasia including kidney hyperplasia, but do not develop PKD (101). These results suggest that the overexpression of Cux1 is insufficient to develop PKD in mice.

To test the hypothesis that Cux1 is required to develop PKD, we generated a conditional Pkd1 deletion mouse line (Pkd1<sup>CD</sup>) that also carries a targeted deletion in the Cux1 gene (Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD). While the Pkd1<sup>CD</sup> mice developed severe PKD by postnatal day 7 (P7) (152), mice which were Pkd1 homozygous null and heterozygous for Cux1 $\Delta$ HD (Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD<sup>+/-</sup>) were healthy and had fewer renal cysts even at 3 weeks of age. Whereas most of the Pkd1<sup>CD</sup> mice died within 2-3 weeks of age, the Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD<sup>+/-</sup> mice had a longer life span and lived for about 4 weeks of age. The cyclin kinase inhibitor p27, which is a transcriptional repression target of Cux1, was up-regulated specifically in the collecting ducts of the double mutant mice where Pkd1 had been deleted. Moreover, this was associated with a marked decrease in cell proliferation. Reduced gene dosage of Cux1 together with the resultant re-expression of p27 may explain the reduced cystic severity as well as the longer life span in these double mutant mice.

Our previous results show a reduction in nuclear cathepsin-L, a protease that cleaves the full length form of Cux1 in ADPKD and Pkd1 null kidneys (87). This in turn leads to the accumulation of the full length Cux1 and increased cell proliferation. Because Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD heterozygous mice retain a normal Cux1 allele, the reduced cathepsin-L likely results in the accumulation of full length Cux1 and subsequent progression of PKD in these animals. Taken together, our results suggest that Cux1 gene dosage influences the progression of cystic disease in an ADPKD mouse model.

## Experimental procedures

### Animals

All animal protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. The University of Kansas Medical Center is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

### Pkd1<sup>CD</sup> mice

The Pkd1<sup>CD</sup> mouse line has been described previously (152). To generate this mouse line, heterozygous Pkd1<sup>cond</sup> mice were crossed with Hoxb7/Cre mice. Hoxb7/Cre is expressed in the mesonephric duct of the kidney as early as embryonic day 9.5 and thereafter, in the mesonephric duct derivatives of the kidney, which include the collecting duct and the ureteral epithelia (148). The Pkd1<sup>cond</sup> mouse line has *loxP* sites flanking exons 2 through 4 of the murine *Pkd1* gene (147). A cross between the Pkd1<sup>cond</sup> and Hoxb7/Cre mice yield the Pkd1<sup>CD</sup> mice which have a conditional deletion of the Pkd1 gene in their renal collecting ducts. These mice developed severe PKD by postnatal day 7 (P7) and died between P15 and P21 (152).

### cux/CDP<sup>ΔHD/ΔHD</sup> mice

This mouse model has been described previously (122). These mice were generated using a homologous gene targeting method in mice which resulted in a deletion of the C-terminal domain of the Cux1 protein, a loss encompassing its homeodomain and repression domain. This deletion leads to the loss of nuclear localization of Cux1 and the mutant protein is localized in the cytoplasm (123). Mice homozygous for the *cux/CDP<sup>ΔHD/ΔHD</sup>* mutation displayed stunted growth, wavy whiskers and cachexia due to muscle wasting. They also had difficulty thriving

and gained little weight. Heterozygous  $cux/CDP^{\Delta HD/\Delta HD}$  mice are indistinguishable from the wild type mice. The  $cux/CDP^{\Delta HD/\Delta HD}$  mice will be referred as Cux1 $\Delta$ HD hereafter.

#### Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD mice

This mouse line was generated by first breeding the heterozygous Pkd1<sup>cond</sup> mice with the Cux1  $\Delta$ HD heterozygous mice. The F1 littermates from this cross, which were heterozygous for both the Pkd1<sup>cond</sup> and Cux1 $\Delta$ HD genes, were crossed onto the Hoxb7/Cre mouse line. Another brother-sister cross was set up between the F1 generation of the latter cross to generate Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>+/-</sup> and Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>-/-</sup> mice.

#### Gt(ROSA)26Sor<sup>tm4(ACTB-tdTomato,-EGFP)Luo</sup>/J (mTmG) mice

The mTmG mice have a TomatoGFP cassette inserted into the Rosa26 locus. The Tomato cassette (mT) is flanked by two loxP sites and followed by a polyadenylation signal. The GFP cassette (mG) is present after the second loxP site. A chicken actin promoter (pCA) drives the constitutive expression of the Tomato cassette in these mice resulting in the presence of red fluorescence in all tissues. In the presence of a tissue-specific Cre recombinase, the Tomato cassette is deleted and GFP is expressed. This double fluorescent reporter system can therefore be used for tracking the activity of the Cre mouse line by following a change in red-to-green fluorescence (153).

#### Characterization of cystic phenotype

We analyzed Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD mice at postnatal day 0 (P0, newborn), P15 and P23 stages. Newborn mice were decapitated. Mice at P15 and P23 were anesthetized using isoflurane and cervical dislocation was done. Kidneys were harvested from all mice. Harvested kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Mid-sagittal sections

(5µm) of cystic kidneys, stained with Haematoxylin and eosin (H & E), were utilized to stage the cysts as described previously (87, 154). Epithelial cells lining each cyst were counted. Cysts with 50 cyst-lining epithelial cells were defined as small cysts and 51-200 cyst-lining epithelial cells as medium cysts. Large cysts were defined as having more than 200 cyst lining epithelial cells. The cystic index (ratio of cystic area in the kidney to the total kidney area) was measured in H & E stained cystic kidney sections using ImageJ (NIH) software.

### Immunofluorescence (IF)

Kidney sections were treated with 1M ammonium chloride to quench autofluorescence. Sections were washed in PBST and blocked in 10% normal goat serum (NGS) or 10% normal horse serum (NHS) for 1 hour at room temperature. Sections were treated with rabbit anti-Cux1 (1:50, Santa Cruz), mouse anti-PCNA (1:3000, Sigma), rabbit anti-p27 (1:100, AbCam) primary antibodies for overnight at 4<sup>0</sup>C. Biotinylated goat anti-rabbit (1:400) secondary antibody was used to detect Cux1 and p27. The sections were then subsequently incubated with FITC-avidin (5µg/ml, Vector). PCNA antibody was detected using a horse anti-mouse Texas Red antibody (Vector, 1:400). To identify collecting ducts, kidney sections were labeled either with Pan-cytokeratin (1:400, Sigma) or dolichus biflorus agglutinin (DBA). Sections were washed in PBST after the antibody treatments, mounted with Vectashield medium with DAPI (Vector) and images were captured with an Optronics Magnafire digital camera.

### Western blotting

Whole tissue lysates were prepared from frozen kidneys. Protein (50ug) was loaded onto 4-15% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% milk in PBST. After blocking, membranes were probed with rabbit anti-Cux1 (1:50, Santa Cruz),



mouse anti-p27 (1:200, AbCam), or mouse anti- $\beta$ -tubulin (1:5000, Sigma) primary antibody followed by PBST washes and HRP-peroxidase secondary antibody (1:10,000) application.

#### TUNEL assay

Sections were processed for Terminal deoxynucleotid transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) with the TUNEL apoptosis detection kit (Upstate biotechnology) according to manufacturer's instructions. TUNEL- labeled sections were counterstained with DAPI, cover-slipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera.

#### Statistics

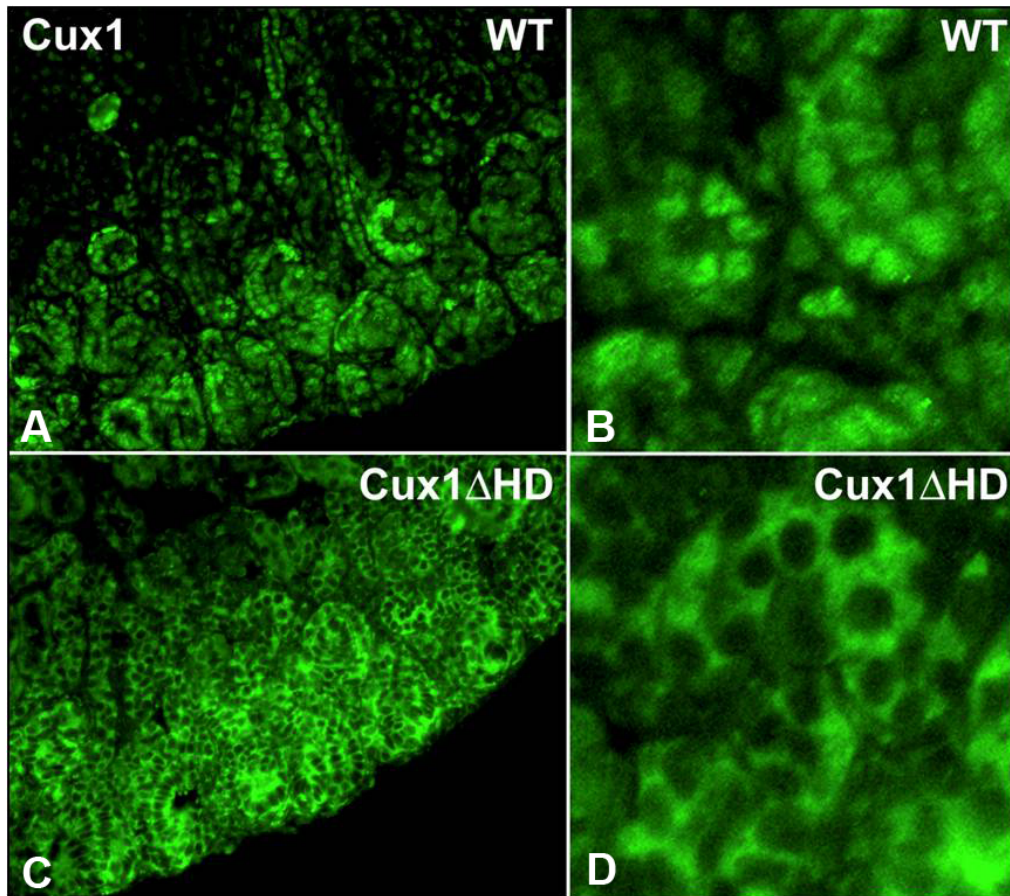
Two-tailed t- tests were performed in all statistical studies.

#### Results

We have previously reported the increased expression of Cux1, a cell cycle regulatory gene, in different PKD mouse models, as well as in humans with ADPKD (87, 124). The Cux1 transgenic mouse which overexpresses Cux1, presented with multiorgan hyperplasia including kidney hyperplasia, however, these mice did not develop PKD (101). These results led us to conclude that the overexpression of Cux1 by itself is insufficient to develop PKD. In order to test the hypothesis that Cux1 is required to develop PKD, we generated the Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD mouse model. These mice have a deletion of the Pkd1 gene in the collecting ducts of their kidneys together with a targeted deletion in the Cux1 gene.

The loss of the NLS of mutant Cux1 $\Delta$ HD protein and its presentation in the cytoplasm has been described previously in mouse embryonic fibroblasts (123). Here we show the

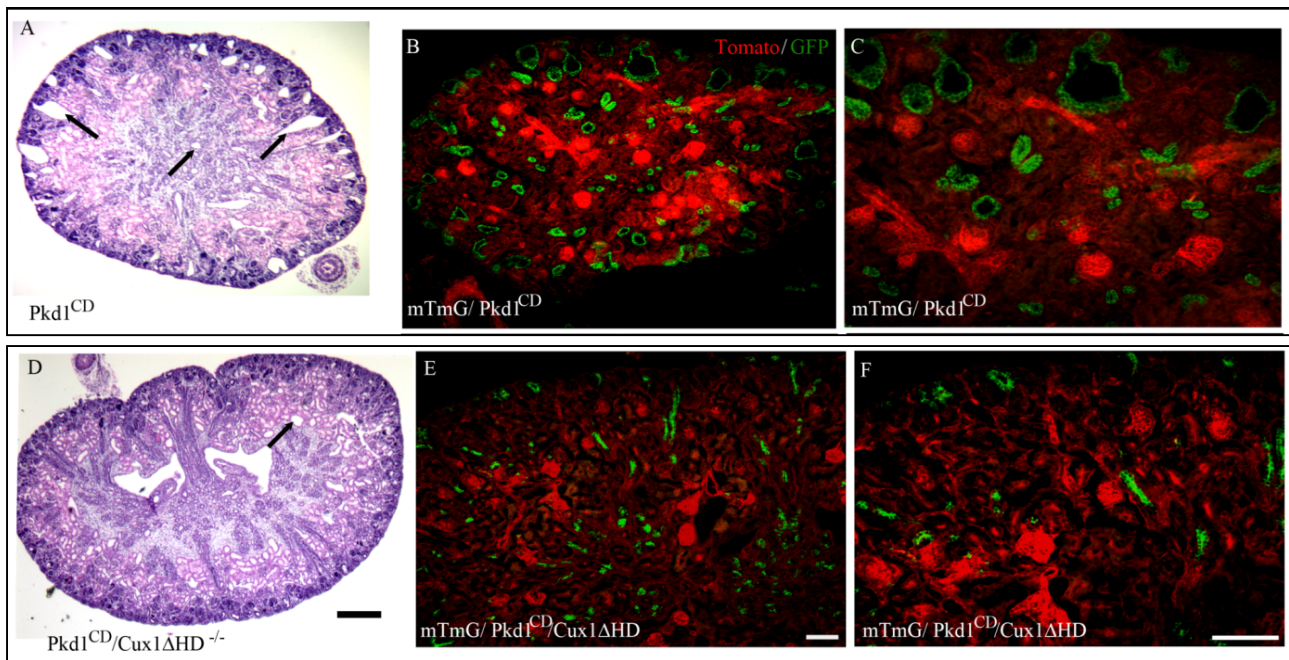
exclusion of Cux1 $\Delta$ HD from the nuclei in Cux1 $\Delta$ HD<sup>-/-</sup> mouse kidneys. Compared to the nuclear localization of Cux1 in the wild type kidneys (Figure 3 A-B), the mutant protein is restricted to the cytoplasm in Cux1 $\Delta$ HD homozygous null kidneys (Figure 3 C-D).



**Figure 3-1 Loss of NLS leads to the exclusion of mutant Cux1 $\Delta$ HD protein from the nucleus.** Kidney sections from wild type and Cux1 $\Delta$ HD homozygous mice were stained with Cux1 antibody. A and B: Cux1 localizes to the nucleus in wild type kidneys. C and D: Due to the loss of its NLS, Cux1 $\Delta$ HD mutant protein is sequestered outside the nucleus. Cux1 staining can be seen in the cytoplasm.

### Cystic dilations are fewer or absent in newborn Pkd1<sup>CD</sup>/Cux1ΔHD<sup>-/-</sup> kidneys

Newborn Pkd1<sup>CD</sup>/Cux1ΔHD<sup>-/-</sup> mice showed none or very few cystic dilations in their kidneys compared to newborn Pkd1<sup>CD</sup> kidneys (Figure 3-2 A and D). We confirmed Hoxb7/Cre activity in both the Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD mice by crossing them with the membrane-targeted Tomato/Green Fluorescent Protein (mT/mG) double fluorescent reporter mouse strain (*Gt(ROSA)26Sor<sup>tm4</sup>(ACTB-tdTomato,-EGFP)<sup>Luo</sup>/J*, Jackson Laboratories, Bar Harbor, ME). These reporter mice have Tomato and GFP cassettes inserted into their Rosa26 locus. Before Cre recombination, a chicken actin promoter drives the constitutive expression of the membrane tagged Tomato protein (153). Tissue specific Cre recombination leads to the deletion of the Tomato cassette and the expression of GFP in the tissue of interest leading to a change from red- to green fluorescence. Since we used the Hoxb7/Cre recombinase, which is active in the ureteric bud derivatives of the kidney, normal and cystic collecting ducts in these mice expressed GFP after Cre recombination, while all the other cells expressed the red Tomato protein (Figure 3- 2 B-C and E-F).



**Figure 3-2 Loss of functional Cux1 in Pkd1<sup>CD</sup>/Cux1ΔHD homozygous mice results in the absence or reduction of renal cysts.** A: Bright field image of a newborn kidney from a Pkd1<sup>CD</sup> mouse. Microscopic cyst dilations (arrows) can be seen in this kidney. D: Bright field image of a newborn kidney from a Pkd1<sup>CD</sup>/Cux1ΔHD<sup>-/-</sup> mouse. Very few and comparatively small cystic dilations were seen in these kidneys (arrows). B-C and E-F: A merge of Tomato (red) and EGFP fluorescence is shown in the kidneys from mTmG/ Pkd1<sup>CD</sup> (B-C) and mTmG/Pkd1<sup>CD</sup> Cux1ΔHD<sup>-/-</sup> (E-F) mice. Cysts (in B and C) and collecting ducts show a change in red-to-green fluorescence while all the other structures continue to show red fluorescence. A and D, scale bar= 200μm. B-C and E-F, scale bar=100 μm.

### Morphological evaluation of the Pkd1<sup>CD</sup>/ Cux1ΔHD mice

Since the Cux1ΔHD homozygous mice had a short life span and died within a week of age, we focused our studies mainly on the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice.

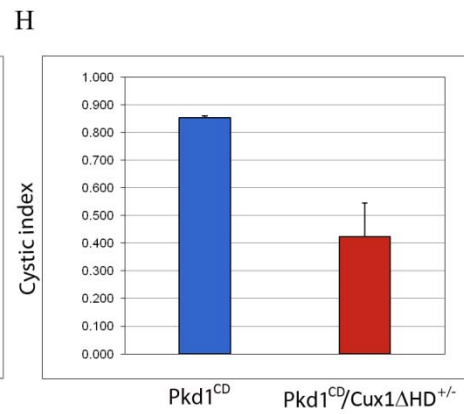
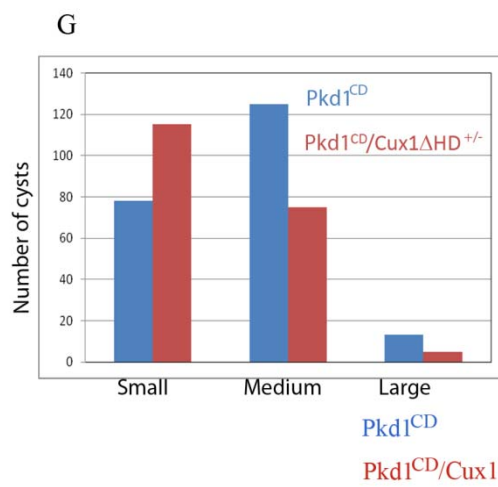
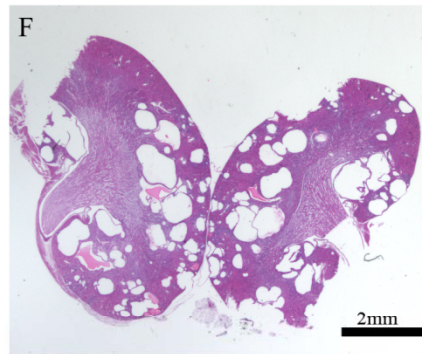
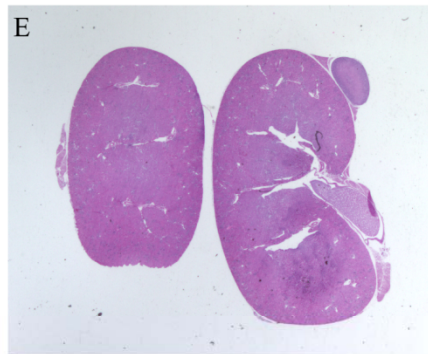
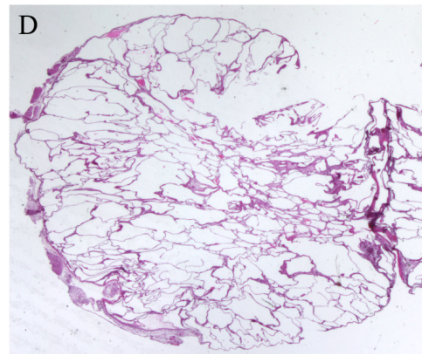
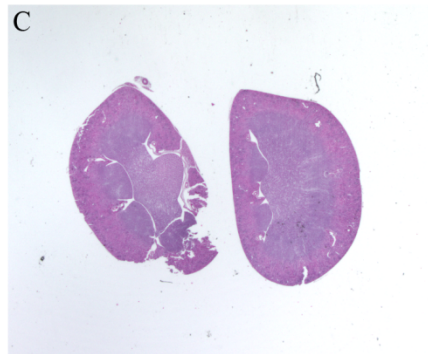
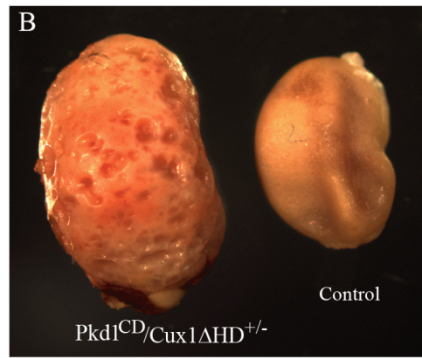
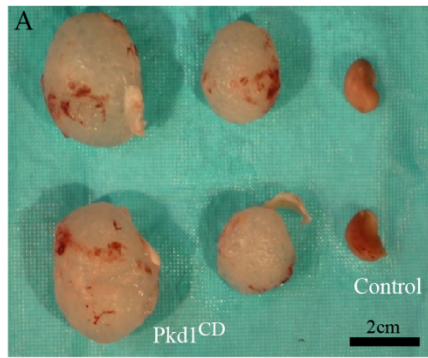
When we analyzed Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice at postnatal day 23, these mice appeared healthy and did not show any signs of PKD, such as enlarged flanks, as we had observed in the Pkd1<sup>CD</sup> mice even at P7. The gross appearance of the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys revealed the presence of cysts. However, it was clear from superficial analysis that these kidneys had less

cystic fluid and more normal renal parenchyma compared to the Pkd1<sup>CD</sup> kidneys at P15 (Figure 3-3 A-B). Morphological analysis of the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys at P23 showed the presence of cystic dilations (Figure 3-3 F). Compared to the non-cystic kidneys from age-matched control mice, Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were slightly larger (Figure 3-3 B and E). Kidneys from Pkd1<sup>CD</sup> mice were crowded with cystic tissue by P15 (Figure 3-3 D). In contrast, Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys at P23 presented with less cystic tissue and more normal renal parenchyma (Figure 3-3 F).

Renal cysts in P23 Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were classified according to their developmental stages and were compared to the renal cysts from P15 Pkd1<sup>CD</sup> mice (refer experimental procedures). Cystic kidneys in P23 Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were mainly composed of small and medium sized cysts. Very few large stage cysts were also seen in these kidneys. In comparison to the Pkd1<sup>CD</sup> cystic kidneys, Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys showed an increase in the number of small cysts. Medium and large cysts were fewer in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to the Pkd1<sup>CD</sup> cystic kidneys (Figure 3-3 G).

The percentage of cystic area in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys was analyzed by measuring the cystic index, which is the ratio of cystic area to the total kidney area (Figure 3-3 H). As observed morphologically, we observed a significant reduction in cystic index in the P23 Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to that of the P15 Pkd1<sup>CD</sup> kidneys.





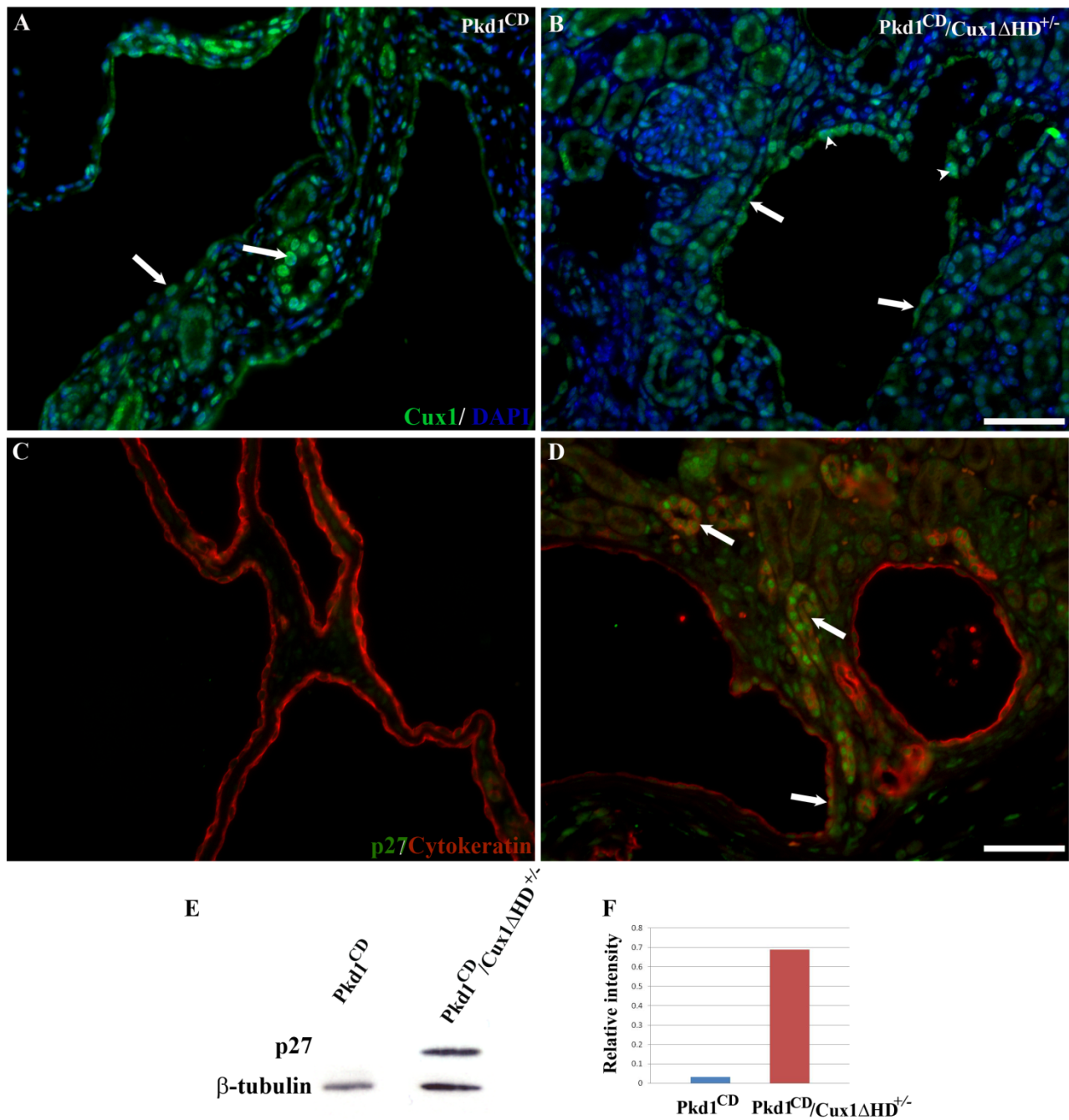
**Figure 3-3 Morphological evaluation of the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice** A: Gross appearance of severely cystic Pkd1<sup>CD</sup> kidneys at P15 together with kidneys from age-matched non-cystic littermates. B: Gross appearance of a cystic kidney from a Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mouse at P23. Control kidneys from age-matched non-cystic littermates are also shown. C and D: Bright field images of non-cystic control (C) and Pkd1<sup>CD</sup> (D) kidneys at P15. E and F: Bright field images of non-cystic control (E) and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (F) kidneys at P23. A, scale bar= 2cm. C-F, scale bar=2mm. G: Cysts were classified according to their developmental stages. Small cysts had up to 50 cyst-lining epithelial cells. Cysts which had 51-200 cyst-lining epithelial cells were defined as medium cysts. Large cysts were lined with more than 200 cyst-lining epithelial cells. Compared to the Pkd1<sup>CD</sup> kidneys (n=4), the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (n=2) had more number of small cysts. Intermediate and advanced stage cysts were fewer in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to the Pkd1<sup>CD</sup> kidneys. H: Cystic index of kidneys from Pkd1<sup>CD</sup> (n=4) and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (n=2) mice at P15 and P23 are shown. Cystic index was lower in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to the Pkd1<sup>CD</sup> kidneys (p value 0.008).

Reduced Cux1 gene dosage correlated with the expression of p27 in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys.

Ectopic expression of Cux1 was seen in the normal tubules and cystic epithelium of the Pkd1<sup>CD</sup> kidneys. Localization of Cux1 was restricted to the nucleus in these kidneys (Figure 3-4 A). In contrast, the ectopic expression of Cux1 that was seen in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> cystic kidneys localized to both the nucleus and cytoplasm (Figure 3-4 B).

Since the CKIs p27 and p21 are transcription repression targets of Cux1, we analyzed the expression pattern of these CKIs. We did not see any change in the expression pattern of p21 in the Pkd1<sup>CD</sup> or Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> cystic kidneys (data not shown). Kidney sections from Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were co-labeled with p27 and cytokeratin. Cytokeratin was used to mark collecting ducts. As previously described (22), a significant reduction in p27 was seen in the Pkd1<sup>CD</sup> kidneys by immunofluorescence and western blot analysis (Figure 3-4 C and E-F). Cytokeratin positive, collecting duct-derived cysts showed little or no expression of

p27 in Pkd1<sup>CD</sup> kidneys (Figure 3-4 C). In contrast, p27 was re-expressed in the cytokeratin positive, cyst-lining cells of Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys (Figure 3- 4 D). Western blot analysis confirmed the increase in p27 levels in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to the Pkd1<sup>CD</sup> kidneys (Figure 3-4 E-F).





**Figure 3-4 Reduced Cux1 gene dosage correlated with the expression of p27 in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys.** A-B: Kidney sections from Pkd1<sup>CD</sup> (A) and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (B) mice were labeled with Cux1 (green) and merged with DAPI (blue) staining. Pkd1<sup>CD</sup> kidneys showed the presence of nuclear Cux1 (arrows in A). Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys showed both nuclear (arrowheads in B) and cytoplasmic (arrows in B) staining of Cux1. C-D: Kidney sections from Pkd1<sup>CD</sup> (C) and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (D) mice were co-labeled with p27 (green) and cytokeratin (red). These images were merged together. Cytokeratin positive cysts in Pkd1<sup>CD</sup> kidneys did not show p27 expression (C). Some cytokeratin positive cysts and many normal tubules (arrows) showed p27 expression (D). A-D Scale bar= 50μm. E: Whole kidney lysates were prepared from P15 Pkd1<sup>CD</sup> and P23 Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice. A western blot analysis was done on these lysates with antibodies raised against p27 and β-tubulin (loading control). We saw an increase in the expression of p27 in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys compared to Pkd1<sup>CD</sup> kidneys. F: Relative intensity of p27 bands compared to the loading control is shown.

A decrease in cell proliferation and increase in apoptosis was observed in Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys

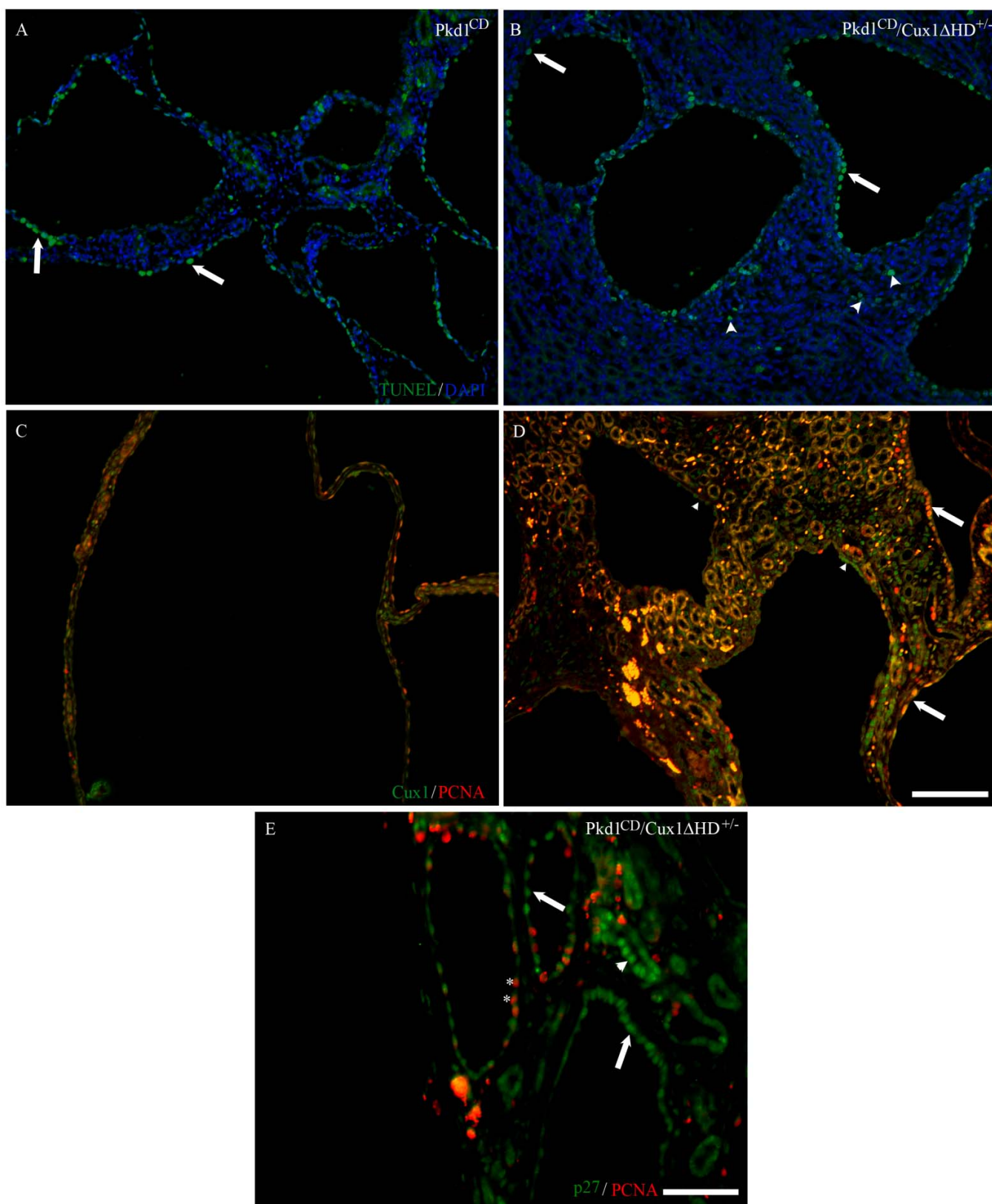
Increased apoptosis and cell proliferation are two pathological features in PKD (7, 142, 144). We analyzed apoptosis by TUNEL assay on Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidney sections. As described previously (152), Pkd1<sup>CD</sup> kidney sections showed an increase in apoptotic cells. Most of the cyst-lining cells were TUNEL positive (Figure 3-5 A). TUNEL positive cells were also seen in Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys. As in the Pkd1<sup>CD</sup> kidneys, many of the cyst-lining cells were apoptotic and TUNEL positive cells were also found in some tubules which appeared normal (Figure 3-5 B).

Cell proliferation was analyzed by staining kidney sections with the cell proliferation marker PCNA. These kidney sections were also co-labeled with Cux1. In comparison to the Pkd1<sup>CD</sup> kidneys, which showed an increase in cell proliferation (Figure 3-5 C), cell proliferation was reduced in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys. Nevertheless, there were proliferating cells

lining Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> renal cysts. Cux1 and PCNA co-localized in many cyst-lining cells, however, there were some cells which were positive for Cux1 but negative for PCNA (Figure 3-5 D).

Increase in the expression of p27 is associated with a decrease in cell proliferation in Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys.

Kidney sections from the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were co-labeled with p27 and PCNA antibodies. Increased p27 expression was seen mainly in the cyst-lining cells of smaller cysts. Co-localization of p27 and PCNA was seen only in a subset of cyst-lining cells. Small cystic dilations were devoid of PCNA while they continued to express p27. At the same time, we also observed PCNA positive cells which were negative for p27 (Figure 3-5 E).



**Figure 3-5 Analysis of cell proliferation and apoptosis in Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys.** A -B: TUNEL assay (green) was done on Pkd1<sup>CD</sup> (A) and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> (B) kidney sections and the images were merged with DAPI (blue) staining. Increased apoptosis was seen in both Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys. Most of the cyst-lining epithelial cells (arrows in A and B) were positive for TUNEL staining. Some normal renal tubules in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys were also TUNEL positive (arrowheads in B). C-D: Kidney sections from Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were co-labeled with Cux1 (green) and PCNA (red), a cell proliferation marker. Merged images are shown. Increased cell proliferation was seen in the Pkd1<sup>CD</sup> kidneys (C). A decrease in cell proliferation was seen in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys as seen by PCNA staining (arrows in D). Some cyst-lining cells were positive for Cux1 but not for PCNA (arrowheads in D). A-D, scale bar= 100μm. E: Kidney sections from Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mice were stained with p27 (green) and PCNA (red). Many of the cyst-lining and normal epithelial cells were negative for PCNA staining. This pattern also correlated with an increase in the expression of p27 in these cells (arrows). Few cells which were positive for PCNA, did not show p27 labeling (\*). Co-localization of p27 and PCNA were seen in a very few cells (arrowheads). E, scale bar=50μm.

## Discussion

ADPKD is a systemic disorder characterized primarily by renal cysts and several extra-renal manifestations (2). At a molecular level, ADPKD shows increased cell proliferation and apoptosis of the cyst-lining epithelia, increased fluid secretion, extracellular matrix abnormalities and epithelial polarity defects (11). Several functions have been described for polycystins, mutations in which are responsible for ADPKD. Previous studies show that polycystins induce the expression of the CKI, p21, by inhibiting cdk2. This upregulation of p21 leads to cell cycle arrest at G0/G1 (48). However, in the case of ADPKD, where either of the polycystins are mutated, this cell cycle arrest may not occur which can lead to increased cell proliferation.

There is debate about which molecular pathogenic event happens first in order to initiate cystogenesis. However, increased cell proliferation is a hallmark feature of PKD kidneys. Increased cell proliferation can be explained by the involvement of polycystins in the cell cycle

and also by the dysregulated expression of many cell cycle related genes such as EGF, TGF, the mTOR pathway, (11) and also Cux1, on which we focused our research.

We generated a double mutant mouse model by deleting Cux1 and Pkd1. The deletion of Pkd1 was specifically restricted to the renal collecting ducts. Analysis of newborn homozygous double mutant mice ( $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{-/-}$ ) showed a complete absence or significantly fewer renal cysts in comparison to newborn  $\text{Pkd1}^{\text{CD}}$  mice. Compared to their homozygous counterparts ( $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{-/-}$ ), newborn heterozygous  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  mice had more cysts, but fewer than  $\text{Pkd1}^{\text{CD}}$  kidneys. These results demonstrate that even though there is a complete functional loss of Cux1 that reduces cell proliferation to an extent, cysts can still form.

Heterozygous  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  kidneys at P23 showed a significant reduction in cystogenesis. While cystic kidneys from the  $\text{Pkd1}^{\text{CD}}$  mice were crowded with medium and large cysts, the  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  kidneys showed primarily small cysts and preservation of normal renal parenchyma. The observation of more small cysts in the  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  kidneys suggests that the cysts that formed were not progressing as fast as cysts in the  $\text{Pkd1}^{\text{CD}}$  kidneys. A reduction in Cux1 gene dosage was unable to prevent cysts from forming, however, it slowed down the process of cystogenesis probably through a reduction in cell proliferation.

Cell proliferation was reduced in the  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  kidneys compared to the  $\text{Pkd1}^{\text{CD}}$  kidneys. Many of the non-proliferative cells in the  $\text{Pkd1}^{\text{CD}}/\text{Cux1}\Delta\text{HD}^{+/-}$  kidneys also showed increased expression of p27, a CKI transcriptionally repressed by Cux1. The increase in the expression of p27 was seen in many normal tubules as well as cysts and noticeably more in smaller cystic dilations. Western blot analysis of these kidneys also confirmed the increased

expression of p27. In comparison to the Pkd1<sup>CD</sup> kidneys, p27 was up-regulated in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys.

The analysis of apoptosis in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys showed similar results as observed during late stage cystogenesis in the Pkd1<sup>CD</sup> mice. Most of the cyst-lining cells expressed Cux1 and were TUNEL positive. The TUNEL positive cyst-lining cells may be explained by the opposing signals that these cells receive: 1) a mutation in the Pkd1 gene which signals the cells to proliferate probably as a compensatory mechanism, 2) an increase in p27 which gives them a signal to arrest at G0/G1. These contrasting signals may force the cyst-lining cells and the normal collecting duct epithelia which has a deletion in the Pkd1 gene to undergo apoptosis.

Results from the Pkd1<sup>CD</sup> and Pkd1<sup>CD</sup>/Cux1ΔHD mouse models can be summarized as follows. In the Pkd1<sup>CD</sup> mouse model, there is an increase in the expression of Cux1 owing to the mutation of the Pkd1 gene in renal collecting ducts. This increase in Cux1 leads to an inhibition of p27, allowing them to progress through the cell cycle. This in turn leads to an increase in cell proliferation leading to severe PKD by P7.

The Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> mouse model has a targeted deletion in Cux1 together with a conditional deletion of the Pkd1 gene in collecting ducts. The Cux1ΔHD mutation renders the mutant Cux1 protein unable to enter the nucleus. A reduction in the dosage of functional Cux1 results in the de-repression of p27 and hence an arrest of cells in the G0/G1 phase of the cell cycle. This leads to the decrease in cell proliferation observed in the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys. Owing to the innate Pkd1 mutation in the collecting ducts, cystogenesis occurs in these kidneys. However, the de-repression of p27 acts as a break, slowing down one of the important

pathogenic aspects in PKD, cell proliferation. Taken together, our results suggest that by decreasing cell proliferation, we can partially rescue PKD in an ADPKD mouse model. Rather than preventing cystogenesis altogether, this mechanism considerably slowed down the process of cyst development.

Even though the reduction in Cux1 gene dosage slowed down the process of cystogenesis in  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice, these mice exhibited an increase in flank size typical of PKD by P29, and died soon thereafter. Our previous results suggest a role for the nuclear isoform of cathepsin-L in ADPKD. Cathepsin-L cleaves the full length Cux1 to generate shorter isoforms of Cux1. A reduction in cathepsin-L and a concomitant accumulation of the full length Cux1 was observed in ADPKD patients, as well as in the  $Pkd1$  null mouse model (87). We speculate that the reduction in cathepsin-L, and the resulting accumulation of the full length Cux1 from the normal Cux1 allele in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  animals eventually leads to severe PKD.

One of the mechanisms through which polycystins exert their regulatory role in cell cycle is by signaling through the JAK-STAT pathway. It has been shown that polycystin 1 binds to Jak2 and regulate STATs 1, 3 and 6 (50-51). Research in the obesity field shows that cathepsin-L is also regulated by STAT3 (128). These results suggest a role for polycystins in regulating Cux1 through STAT3 and cathepsin-L. A detailed study evaluating the expression pattern and levels of cathepsin-L and STAT3 in  $Pkd1^{CD}$  and  $Pkd1^{CD}/Cux1\Delta HD$  may give conclusive information in this regard.

## Chapter Four

### Conclusions and future directions

A potential role for the homeodomain protein Cux1 in PKD was established by the observations that it was ectopically expressed in several mouse models of PKD and also in ADPKD patients (87-88, 124). In order to determine whether ectopic expression of Cux1 induces cysts in mice, Ledford *et al* made a transgenic mouse model which overexpressed Cux1. The global overexpression of Cux1 in these mice led to multiorgan hyperplasia including kidney hyperplasia, however, they did not develop PKD (101). This suggested that the overexpression of Cux1 is not enough to induce cystogenesis.

As a next step in elucidating the role of Cux1 in PKD, I set out to test the hypothesis that Cux1 is required for cystogenesis or cyst progression in ADPKD. Because Pkd1 null mice die embryonically (60), we first generated an ADPKD mouse model with the conditional inactivation of the Pkd1 gene in renal collecting ducts (Pkd1<sup>CD</sup>). The Pkd1<sup>CD</sup> mice were then used to generate a double mutant mouse model which had a homozygous deletion in the Pkd1 gene together with a targeted deletion in the Cux1 gene (Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD).

The Cux1 $\Delta$ HD mouse model which I used in generating the Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD mice had a 4kb deletion in the Cux1 gene. This deletion in the Cux1 gene generates a mutant Cux1 $\Delta$ HD protein which has a loss of its C-terminal domain encompassing the homeodomain, repression domain and more particularly a loss of its nuclear localization sequence (NLS). The loss of the NLS in the Cux1 $\Delta$ HD mutant protein makes it functionally inactive since Cux1 is a transcription factor which functions in the nucleus. The Cux1 $\Delta$ HD homozygous null mice do not show any



renal phenotype. However, they display stunted growth, have difficulty thriving, have increased susceptibility to bacterial infections and most of them die within a week after birth. The Cux1 $\Delta$ HD heterozygous mice were indistinguishable from their wild type littermates and did not have any developmental abnormalities (122-123).

First, we analyzed the Cux1 $\Delta$ HD null mice carrying a collecting duct specific deletion in the Pkd1 gene (hereafter Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>-/-</sup>). These mice had a complete loss of function of Cux1 since they had lost both the normal copies of Cux1. Analysis of newborn Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>-/-</sup> mice showed either an absence of renal cysts or if cysts were present they were very few and smaller compared to the renal cysts in the Pkd1<sup>CD</sup> mice. This suggested that the loss of Cux1 has slowed down the cystogenesis process. However, the presence of renal cysts in these mice show that Cux1 is not required for cyst initiation.

Because of the perinatal lethality of the Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>-/-</sup> mice we focused our studies mainly on Cux1 $\Delta$ HD heterozygous mice carrying a collecting duct specific deletion in the Pkd1 gene (hereafter Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>+/-</sup>). As the genotype implies, these mice have one normal copy of the Cux1 gene and the protein transcribed from this allele can enter the nucleus. The other Cux1 allele generates a mutant Cux1 $\Delta$ HD protein which gets excluded from the nucleus because of the loss of its NLS, leaving it as a non-functional protein.

Analysis of the newborn Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>+/-</sup> kidneys showed the presence of renal cysts, however, they were smaller and fewer compared to the renal cysts in the Pkd1<sup>CD</sup> mice. This suggests that the reduced gene dosage of Cux1 in these mice slowed down the cystogenesis process. Further analysis of the Pkd1<sup>CD</sup>/ Cux1 $\Delta$ HD<sup>+/-</sup> mice at postnatal day 23 (P23) showed that these mice had fewer renal cysts and a preservation of normal renal parenchyma compared to the

Pkd1<sup>CD</sup> kidneys which were crowded with cystic tissue even at P15. Consistent with the reduction of the gene dosage in Cux1, the Pkd1<sup>CD</sup>/ Cux1ΔHD<sup>+/-</sup> kidneys showed an ectopic expression of p27, which is a transcriptional repression target of Cux1. The ectopic expression of p27 in these kidneys led to a decrease in cell proliferation resulting in the slow progression of the disease.

Even though ADPKD is considered an adult onset disease, cysts as big as 7mm are observed at birth in children with ADPKD. A recent study by Grantham *et al* measured the diameter, volume and growth rates of individual cysts in adult ADPKD patients by magnetic resonance imaging over a period of 3 years. This study showed that the growth rate of cysts in these adult patients was far less than the rate which is required to develop a cyst with a 7mm diameter at birth. This suggested that the in utero environment may be very conducive for cystogenesis and therefore many of the cysts that are detectable at birth may have gone through exuberant growth rates *in utero* (18). Therefore, treating ADPKD patients in the *in utero* period could be a promising approach in order to effectively halt the cystogenesis program. However, at present, it is impractical and perhaps unethical. Nevertheless, our results with the Pkd1<sup>CD</sup>/ Cux1ΔHD mice show the effectiveness of such treatments since these mice presented with fewer and smaller renal cysts at birth. We speculate that the loss or the reduction in Cux1 gene dosage and the resultant increase in p27 may have put a brake on the exuberant *in utero* cystogenesis process by decreasing cell proliferation.

Another CKI, p21 is also a transcriptional repression target of Cux1 (118). We did not see any change in the expression of p21 in the Pkd1<sup>CD</sup>/ Cux1ΔHD<sup>+/-</sup> kidneys. One possible explanation is that the levels of p21 are normally downregulated very early on during kidney development. Therefore, p21 may have an effect in reducing cell proliferation in the

Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys during early kidney development. We cannot exclude this possibility since we have not studied the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> kidneys embryonically.

Nevertheless, p21 is a very appealing target in reducing cell proliferation in PKD since polycystins also up-regulate the levels of p21 thereby decreasing cell proliferation (48-49). However, abundant evidence suggests that p27 may play a more important role in decreasing cell proliferation than p21. For example, inactivation of p21 in mice showed no developmental defects suggesting that there may be other pathways which can compensate for the loss of p21 (127). In contrast, inactivating p27 has a major impact as it results in hyperplasia and an increase in the size of many organs including kidneys showing the importance of p27 in decreasing cell proliferation (126).

Additional evidence for the importance of p27 in decreasing cell proliferation in PKD comes from the study by Alcalay *et al* (87). They generated a double mutant mouse model by crossing a Cux1 mutant (Cux1ΔCR1) to the cpk mouse model of PKD. This resulted in an aggravation of cystic disease in the cpk mouse when both the copies of Cux1 were mutated. However, mice which were heterozygous for the Cux1ΔCR1 mutation and homozygous for the cpk mutation did not show a significant difference in the size of their kidneys compared to the cpk cystic kidneys. They noticed that there was an ectopic expression of p27 in these kidneys but not of p21. This also suggested that it was the ectopic expression of p27 and a resultant decrease in cell proliferation which protected these kidneys from an aggravation of PKD (87). My studies with the Pkd1<sup>CD</sup>/Cux1ΔHD<sup>+/-</sup> also point towards p27 as the important player in reducing cell proliferation and thereby partially rescuing PKD in these mice. Together, these results suggest a

paradigm shift in the field of PKD where we show the importance of p27 over p21 in rescuing PKD.

Increased cell proliferation is an important factor in the progression of PKD. Therefore, translational approaches have been aimed at targeting this important pathogenic pathway. Treatment of a PKD mouse model with roscovitine, a small molecule CKI, showed promising results as these mice showed reduced kidney weight to body weight ratio and cysts volumes (155). Roscovitine is a potent inhibitor of cdk2-cyclin E which functions by targeting the G1-S check point in the cell cycle that is regulated on a molecular level by the CKIs p27 and p21. It also inhibits cdk7, cdk9 and cdk5. At a molecular level, roscovitine binds to the ATP-binding pocket of the Cdk. Specifically, roscovitine inhibits cdk2 thereby preventing phosphorylation of Rb and keeping E2F transcription factor in its inactive state (156). MDCK cells which form cystic structures in 3D collagen gels were treated with roscovitine. This also resulted in a decrease in cell proliferation in these cells. Increased cell proliferation in PKD also results from the activation of the B-Raf/MEK/ERK pathway. Oral administration of a MEK inhibitor in a PKD mouse model was able to reduce cystogenesis and kidney enlargement (157). Overall, these studies suggest the importance of targeting the cell cycle pathway in PKD.

Results from the  $Pkd1^{CD}/Cux1\Delta HD$  mice suggest that Cux1 and p27 are promising cell cycle targets for the treatment in ADPKD. If transcription factors become suitable targets for treatment by siRNA knockdown strategies, Cux1 is an important transcription factor that could be targeted in PKD. Our results show that reducing the levels of Cux1 can lead to a considerable reduction in cell proliferation and a partial rescue of PKD.

Our results also show that the reduced gene dosage of Cux1 exerts its effect by the up-regulation of p27. Therefore another strategy will be to use p27 agonists to treat PKD. One of the follow-up studies that we are currently pursuing is to test if forced expression of p27 can lead to a greater or even complete rescue of PKD in the  $Pkd1^{CD}$  mice. In order to do these experiments, a conditional floxed p27 transgenic mouse model (CMV/p27) has to be generated. The conditional p27 transgenic mouse model can then be used to generate a double mutant mouse model by crossing CMV/p27 to the  $Pkd1^{CD/+}$  mice. These double mutant mice will have a simultaneous inactivation of the Pkd1 gene and a forced direct expression of p27 in the collecting ducts of the kidneys. Unlike the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mouse model which had an increase in the expression of p27 because of the reduced Cux1 gene dosage, the  $Pkd1^{CD}/CMV/p27$  double mutant mouse model will have a direct increase in the expression of p27. We speculate that this forced expression of p27 despite the inactivation of Pkd1 may lead to a greater or even complete rescue of PKD in the  $Pkd1^{CD}$  animals. However, one challenge is that the forced expression of p27 starting from E9.5 in the ureteric bud derivatives of the kidneys may also lead to developmental defects of the kidneys since cell proliferation will be greatly inhibited.

Even though the reduced Cux1 gene dosage in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice results in slowing down cystogenesis and disease progression, these mice eventually die of renal failure by around 4 weeks of age. A study by Alcalay *et al* showed that a nuclear form of cathepsin-L, a protease that processes the full length isoform of Cux1, is reduced in ADPKD cells compared to the normal human kidney cells. A reduction in cathepsin-L was also shown in the Pkd1 null mice compared to the age-matched wild type controls (87). A regulatory loop among polycystin-1, STAT3, cathepsin-L and Cux1 has emerged recently. The Polycystin-1 C-terminal tail has been

shown to interact with STAT3. STAT3 has been shown to regulate cathepsin-L (51, 128). This suggests that the dysregulation of PC1 in ADPKD may be directly linked to the increased expression of Cux1 that is observed in the disease. In this model, a mutation in polycystin-1 would lead to a reduction in STAT3 which would result in reduced levels of cathepsin-L and increased accumulation of the full length Cux1 isoform, leading to the repression of p27 and deregulated cell cycle progression.

We speculate that the reduction in nuclear cathepsin-L also occurs in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice. This reduction in cathepsin-L in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  kidneys will result in a reduced proteolytic processing and accumulation of full length isoform of Cux1. The full length isoform of Cux1 functions by transcriptionally repressing p21 and p27. Therefore, the accumulation of full length Cux1 eventually leads to increased cell proliferation and disease progression in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice. However, the reduction to one functional Cux1 allele in the  $Pkd1^{CD}/Cux1\Delta HD^{+/-}$  mice makes this process of Cux1 accumulation slower, thereby slowing down disease progression. Thus, cathepsin-L is another potential target for treatment of ADPKD. An increase in the expression of nuclear cathepsin-L may lead to an increased processing of Cux1, prevent its accumulation, eventually leading to a decrease in cell proliferation.

The  $Pkd1^{CD}$  mouse model that we generated had a collecting duct specific deletion of the  $Pkd1$  gene. From this mouse model, we learned a developmental aspect of PKD. In particular, we chose to delete  $Pkd1$  in renal collecting ducts and not in any other segments of the nephron for the following reason:

Microdissection studies on human kidneys have revealed that cysts in ADPKD can arise from all segments of the nephron including the collecting ducts (5). However, most of the cysts in ADPKD patients develop from collecting ducts (18). Collecting duct cysts are also more numerous and larger than the cysts derived from other tubular segments (138). It is not known why most of the cysts originate from collecting ducts. However, we can speculate some reasons by analyzing the expression pattern of polycystins and some other proteins that are only expressed in the collecting ducts.

Expression studies on human fetal kidneys show that polycystin expression in both cortical and medullary collecting ducts remains until 40 weeks of gestation, while proximal tubules sustain the expression only until 28 weeks of gestation. This pattern of expression suggests that polycystins are required for a longer time and may be more important in the collecting ducts than in other renal tubules. Murine polycystin-1 also shows a strong pattern of expression in the ureteric bud derivatives such as in collecting ducts, papillary ducts and the renal pelvis during the later developmental stages and this expression pattern continued until around 3 weeks after birth (31, 39). Both the human and murine polycystin-1 showed a weak expression in the ureteric bud tips. This suggests that polycystins may not be required for the initial metanephric induction. Since polycystins show a stronger and spatially restricted expression pattern in collecting ducts, when one of the polycystins is mutated, collecting ducts may be more affected than other nephron segments.

Another reason for the predominance of collecting duct cysts may have to do with the urine concentrating function of the collecting ducts. Principal cells in the collecting ducts express the receptor (V2R) for antidiuretic hormone (ADH), also known as arginine vasopressin (AVP). An increase in plasma osmolality leads to the secretion of AVP which binds to its

receptors on the collecting ducts. This leads to the activation of adenylyl cyclase 6 (AC6) which in turn converts ATP to form the second messenger cAMP. The end result of this signaling pathway is the insertion of AQP-2 channels on the apical membrane of the principal cells and water intake into the cell. This signaling pathway, which occurs primarily in the collecting duct cells can lead to an increase in cAMP. cAMP has been shown to have mitogenic effects on ADPKD cells in contrast to its anti-mitogenic effects on normal human kidney cells. Therefore, the presence of V2R on principal cells and its involvement in a signaling pathway which leads to the production of cAMP may make collecting ducts more susceptible to cystogenesis (138).

Inactivation of *Pkd1* in the *Pkd1*<sup>CD</sup> mice was achieved by E9.5 even before metanephric induction occurred. However, we did not see any cystic dilations in the *Pkd1*<sup>CD</sup> mice when we analyzed them between E14.5 –E15.5. This was not surprising since the *Pkd1* null mice (global knockout of *Pkd1*) also did not show any cystic dilations until E15.5. These results suggest that the inactivation of the *Pkd1* gene does not affect the early metanephric induction events.

While the inactivation of *Pkd1* in the *Pkd1*<sup>CD</sup> mice was restricted to the collecting ducts, these mice developed severe cystic disease by P7. Moreover, by P15, the normal renal parenchyma was completely destroyed and no normal renal tubules were seen. How can we account for the loss of other nephron segments here? Even though the mutation is restricted to the collecting ducts, the cysts that arise from them expand by increased cell proliferation. The expansion of these cysts likely resulted in the compression of the adjacent normal renal parenchyma leading to the apoptosis of normal renal tubules. Consistent with this, we saw increased TUNEL labeling in these kidneys.



Two other collecting duct specific PKD mouse models have been generated by Raphael *et al* (158). They inactivated *Pkd1* specifically in the principal cells (PC) of mouse collecting ducts by using an AQP-2 Cre transgenic mouse. The PC-Pkd1 knockout mice did not show any cysts at birth, however, these mice showed cystic dilations at 1 week of age. They had an average life span of 8.2 weeks.

In a separate experiment, the same group also generated another Pkd1 knockout mouse model by conditionally inactivating *Pkd1* in the intercalated cells (IC) of mouse collecting ducts by using a B1 Cre transgenic mouse. In contrast to the PC-Pkd1 knockout mice, the IC-Pkd1 knockout mice showed a mild cystic phenotype. They showed very few cysts even at 8-9 weeks of age. They attributed the phenotypic differences between the PC and IC knockouts to the possibility of ICs lacking the expression of PC1. They also suggested that PC1 may not also be functionally important in the ICs.

Comparing the PC-Pkd1 knockout mouse model to our Pkd1<sup>CD</sup> mouse model, the Pkd1<sup>CD</sup> mouse model had a more severe phenotype compared to the PC-Pkd1 mouse model. We observed microscopic cystic dilations at birth in the Pkd1<sup>CD</sup> mice while no cysts were present in the newborn PC-Pkd1 mouse model. This difference can be explained by the use of different Cre recombinases to inactivate *Pkd1* in these different mouse models. We used the Hoxb7/Cre transgenic mouse model which inactivates *Pkd1* in the mesonephric duct by E 9.5. In contrast, in the PC-Pkd1 mouse model, Raphael *et al* used an AQP-2 Cre which is not active until E18.5. We did not see any cystic dilations in the Pkd1<sup>CD</sup> mice until E 15.5. The Pkd1<sup>CD</sup> mice likely had an active cystogenesis process between E15.5 and birth which resulted in the presence of microscopic cysts in the newborn mice. This is in accordance with the observation that most of

the cysts that we see in children and young adults with ADPKD must have gone through a process of exuberant growth in the fetal environment (18).

The Pkd1<sup>CD</sup> and the PC-Pkd1 mouse models, both had an inactivation of *Pkd1* embryonically. Yet, the Pkd1<sup>CD</sup> mice developed a more severe and rapid disease than the PC-Pkd1 mice. In the PC-Pkd1 mice, *Pkd1* is inactivated at E18.5. The fact that AQP2-Cre is active in these cells suggests that these cells have differentiated at least partly if not completely and have the characteristics of a collecting duct cell. In contrast, the Pkd1<sup>CD</sup> mice suffered a *Pkd1* mutation at E9.5 when these progenitor collecting duct cells were still in the early stages of development. This difference of inactivating *Pkd1* in the progenitor cells versus differentiated cells may have profound effects. Kidney development is characterized by the reciprocal induction events between the collecting duct progenitor cells and the cells in the metanephric mesenchyme. Therefore, the loss of polycystin-1 starting from E9.5 in the collecting duct progenitor cells in the Pkd1<sup>CD</sup> mice may have had an impact on further kidney development. It is possible that the loss of polycystin-1 in these cells may have left them undifferentiated.

Overall, my dissertation work shows that Cux1, a cell cycle regulatory gene is required for cyst progression in ADPKD emphasizing the importance of increased cell proliferation in the pathogenesis of ADPKD. Accordingly, Cux1, cathepsin-L, which is upstream of Cux1, and p27, the downstream target of Cux1, may be possible targets for pharmacological intervention in ADPKD. Our results also show that reducing the gene dosage of Cux1 and thereby reducing cell proliferation embryonically can slow down the cyst initiation process since we see only few and small cysts in the Pkd1<sup>CD</sup>/Cux1 $\Delta$ HD mice. Our results also indicate that p27 is a major player in reducing cell proliferation in PKD. Finally, our results from the Pkd1<sup>CD</sup> mice show that

inactivating *Pkd1* in the progenitor cells can have a profound effect in the progression of PKD compared to the inactivation of this gene in differentiated cells.

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